

Can Hong Kong avoid a future of decline?

As it nears the transfer of its governance to China, Hong Kong is hampered by the consequences of historical short-sightedness. Its technological future depends on substantial investment and continuing freedoms.

Nobody knows for certain what it's going to be like in Hong Kong over the next few years, but pessimists point to two alternative ways in which the 'special administrative region', as it will be known after its handover to China on 30 June, could tumble from its estimable position at eighth in the ranking of trading nations. The least sad of the two pessimistic scenarios is that Hong Kong becomes the Monaco of East Asia—a pleasure garden tolerated as such by the mainland government, its economy based on trade brokering, property and financial speculation, with the dubious charm of a glitzy European monarchy replaced by an altogether weightier executive backed by the Chinese army.

An even worse alternative is that China loses the will to sustain its much vaunted 'one country, two systems' policy for the territory, stamps out freedom of expression and causes the abandonment of the region, and of its six million Chinese inhabitants, by the world's investors. Few inhabitants give this future much credence. China has too much to lose, goes the argument—both in Hong Kong and in Taiwan, whose reintegration with the mainland is very close to China's heart and for which Hong Kong's new administrative arrangements are seen as a prototype.

With such dire possibilities in mind, those more ambitious for the region might well settle for a continuation of the present—an energetic trading post with low-technology manufacturing dependent increasingly on mainland labour, and a gateway to and from expanding mainland markets. But with China depending less and less on it for its traffic with other countries, Hong Kong has to reposition itself. East Asia has other examples of relatively small economies that have embraced high technology to carve themselves a niche. Can Hong Kong sensibly develop in that direction?

Some of the territory's academics appear to believe so. Certainly it would be hard for it to spend less on research and development than the present fraction of a per cent of gross domestic product. In that negative sense, there is plenty of room for growth. If other East Asian countries are a guide, the *laissez-faire* approach of past executives can be expected to change under the new administration. New tax incentives are likely, to encourage traditional as well as recently founded small high-technology companies to fulfil their potential, while the growth in government funds for university research and development (R&D) may be boosted further, and a nascent science park may be launched—first as a virtual entity, then as a physical development.

Such possibilities can be made to sound positively glowing. Nevertheless, a scrutiny of the territory's science base is sobering. Of the handful of universities, three could claim to have a substantial and internationally competitive research capacity: the venerable Hong Kong University, the Chinese University of Hong Kong and the 'young Turk' of the bunch, Hong Kong University of Science and Technology. All three face challenges unique to Hong Kong.

First, one should recall that the striking growth in science and technology in East Asia's tigers has been accomplished with the indispensable help of Chinese returning from the West, especially from

the United States. The development of science in Taiwan, South Korea and even China has depended on such people filling key posts, both in research institutions and elsewhere. Hong Kong is superficially attractive to such people because, by the region's standards, it pays well, and has been recruiting at a time when the West has been a poor source of employment. But whether it can recruit people of the highest calibre and then retain them is another question. There has been some capital investment in new laboratories in recent years, but the total recurrent expenditure on university research over the past decade would not maintain a major US university for a year.

A second profound challenge relates to the territory's education system. One aspect here is the declining linguistic flexibility of the young. English is meant to be widely spoken within schools and universities, but isn't—and other countries with international attractiveness in mind achieve better performance in English amongst the population. Furthermore, the majority of Hong Kong's inhabitants speak Cantonese rather than the *putonghua* (Mandarin) Chinese common elsewhere. Another worry is cultural: as on the mainland, young Chinese emerge from high school with a depressing lack of inclination to question received ideas. That does not limit their flair as entrepreneurs, but it undermines the potential for flourishing science.

A third millstone is history. Hong Kong is already well on the way to casting off cultural and administrative impediments from its colonial past, but it has flourished economically on business short-termism and the pursuit of rapid personal gain. There cannot be many countries that decide, as Hong Kong's executive did as recently as 1983, that research was not to be encouraged in universities and that companies should be left entirely free of a regional strategy in R&D. As a result, Hong Kong now has a depleted proportion of technology-based industries, and a university science research base and infrastructure that is less than ten years old.

Given the wealth of the territory, and the acknowledgement by the new chief executive Tung Chee-Hwa that technology growth and R&D are important factors for its future, Hong Kong's academics have a great deal to play for. But much depends on the new administration, and the mainland government behind it. The latter's attitude to Hong Kong will no doubt be regrettably tempered by a fear of its potentially liberalizing influence on its population, but also by its urgent need to boost the livelihood of China, where poverty is endemic and widespread.

It is to be hoped that China's rulers will be persuaded that Hong Kong merits immediate and long-term science and technological investment. But if that strategy is to have a chance of succeeding, they will need to keep several goals uppermost in mind, over and above the deployment of more of the territory's resources in education, science and engineering: the positive development of independent thinking and expression in academic institutions, continued open access to the international flow of people and ideas, and the avoidance of cronyism at the expense of merit in appointments. Only thus can Hong Kong avoid a future of intellectual and technological decline. □



Research 'could still result in nuclear arms', warn experts

[WASHINGTON] Some of the research being carried out at nuclear weapons laboratories in Russia and the United States should be banned, as it could lead to dangerous new types of nuclear weapons, according to a number of US weapons scientists.

Under the terms of the Comprehensive Test Ban Treaty, US weapons laboratories, which are funded by the Department of Energy, are prohibited from working on the development of new nuclear weapons.

But, according to several scientists, conceptual, computer-based and even experimental work at Lawrence Livermore laboratory in California and at Los Alamos laboratory in New Mexico could eventually lead to the development of nuclear weapons for battlefield use.

They argue, for example, that research into the problem of inducing fusion directly with chemical explosives — either through the production of an intense magnetic field, or directly, by carefully balanced implosion — could lead to 'pure fusion' weapons whose proliferation would be difficult to control.

Existing nuclear weapons need a fission trigger to induce fusion, and their proliferation is controlled chiefly by monitoring who is producing highly enriched uranium and plutonium, both difficult to manufacture.

Among weapons scientists, there is a wide range of views on the feasibility of 'pure

fusion' weapons. Last month, Hans Bethe, the 90-year-old physicist who served as head of theoretical physics on the Manhattan Project, wrote to President Bill Clinton calling for an explicit ban on "all physical experiments, no matter how small their yield, whose primary purpose is to design new types of nuclear weapons" (see *Nature* 387, 330; 1997 and <http://www.fas.org/betheltr.htm>).

Bethe said that "success is unlikely" with pure fusion weapons. But he expressed concern that the US weapons laboratories might nonetheless solve the problem, and would then be unable to keep their solution secret. He called on Clinton to "review this matter personally" to ensure that no unnecessary weapons work was going on in the laboratories.

"There are always temptations in the laboratories," explains Bethe. "I want our country to go on the record that we really do not want further weapons developed. If the president made such a statement, it would influence thinking in the labs." Bethe says he has not been to the laboratories for a couple of years, and does not know what they are up to.

But Ray Kidder, a former senior weapons designer at Lawrence Livermore, says he would be "very surprised" if the laboratories were not making computer calculations of how pure fusion weapons would work, "and if there wasn't some experimental work going on as well". Kidder worked on pure

IMAGE
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REASONS

Bethe: expresses concern that "there are always temptations in the laboratories".

fusion concepts during the previous moratorium on nuclear tests, from 1958 to 1961.

Work on the concept, he explains, is "the best possible training exercise for skills in high-explosive-driven implosions". But Kidder says he agrees with Bethe's letter, and thinks that work pertaining to pure fusion weapons should be stopped.

Exactly how much such work is going on is unclear. Livermore and Los Alamos deny categorically that they are developing new types of nuclear weapons. But, because many scientists doubt the feasibility of pure fusion weapons, the laboratories may feel free to attack the relevant problems without a weapons mandate. As Kidder puts it, "the labs can say they are not developing a weapon, and be truthful about it — and be working to get ignition to occur" by use of high explosives.

Spokesmen for both laboratories declined to comment on the Bethe letter or to discuss classified programmes they might have on obtaining fusion by means of high explosives. "Experts here remain extremely sceptical that pure fusion has any weapon potential," says Jeff Garbersen, a spokesman for Livermore. "We are not developing or trying to develop pure fusion weapons, or any other new nuclear weapon."

Los Alamos is working on an unclassified collaborative project with the Russian weapons laboratory Arzamas-16 to induce momentary magnetic fields of up to 1,500 tesla by imploding an inductor with up to 50

Two alternative routes to a nuclear trigger

[WASHINGTON] The idea of igniting fusion in a deuterium or deuterium-tritium target by means of conventional high-explosives (see above) has long fascinated both those who saw the concept as a potentially limitless energy source and those who viewed it as a quick route to a simple, devastating weapon.

According to US scientists, the latter concept was taken up by Russian weapons scientists in the early 1950s and pursued vigorously at the Lawrence Livermore laboratory during the 1958–61 nuclear test moratorium, under a programme incongruously entitled 'Dove'.

According to Sam Cohen, who worked on the

Manhattan Project, Dove failed in its goal of developing a neutron bomb "for technical reasons, which I'm still not free to discuss". But Ray Kidder, formerly of Lawrence Livermore, says that the United States lost interest in the Dove programme when testing resumed because it was much simpler to use fission-based triggers.

There are two main approaches to driving fusion with high explosives. The simpler one wraps a target in the explosive and detonates it with enough precision, speed and force to cause fusion directly. The second approach uses the explosive to generate momentarily an extremely intense magnetic field, which in turn causes

fusion in the target.

Chemical explosives are ill-suited to triggering fusion, as they are not particularly hot or fast. They offer much lower and less well-controlled concentrations of energy than will the National Ignition Facility at Livermore.

But according to Frank von Hippel, Russian scientists have claimed results falling within "two orders of magnitude of the ignition threshold". Some observers believe such claims to be exaggerated, however, and the best neutron yield reported fall five or six orders of magnitude short of the 10^{19} neutrons needed for a tiny neutron bomb, that would kill people within a 50-metre radius.

C.M.

kg of high explosives. John Shaner, who directs several collaborative projects between the two laboratories, says that they are not at present trying to achieve fusion, but may do so in the future.

In a paper released this month by the Federation of American Scientists, Frank von Hippel says that the laboratories have been pushing for an interpretation of the test ban treaty that would exempt research, development and test of pure fusion devices. Hippel served the Clinton administration as associate director for national security in the Office of Science and Technology Policy.

Last week, von Hippel was in Moscow and unavailable for comment. But a spokesman for Livermore said: "We have technical discussions with the Department of Energy and let them decide what to push for and what not to push for. It is not a Livermore function to make policy."

In testimony to the Congress last year, however, Paul Robinson, the director of the third US weapons laboratory, at Sandia in New Mexico, told the Senate Armed Services Committee that the laboratories needed to employ "various approaches to inertial confinement fusion" to maintain US weapons without testing.

Robinson wants an exemption for Sandia's efforts to achieve fusion by power-pulse-driven X-rays. Chris Paine, an analyst at the Natural Resources Defence Council, says that the other laboratories want exemption for fusion from high explosives.

Weapons experts remain divided on the feasibility of pure fusion weapons. Sidney Drell, deputy director of the Stanford Linear Accelerator Center in California and a regular adviser to the federal government on nuclear questions, doubts that such a weapon would ever work.

"There's a range of views on this, but I don't care if we allow an exemption, as long as we get the test ban treaty [ratified]." He says that, even if some scientists in the laboratories are keen to pursue the weapons, the management at the laboratories has no interest in doing so.

But some scientists say that pure fusion weapons could work. Sam Cohen, who worked with Bethe on the Manhattan Project, and is now an ardent defence 'hawk', contends that Russia already has them — a claim hotly denied by official US sources.

Cohen wants the United States to end its unclassified collaboration with Russia and get on with developing what he calls "the most useful battlefield weapons ever devised".

Ted Taylor, a former weapons designer at Los Alamos who has opposed nuclear weapons for 30 years, says he "applauds Bethe's position". But he says that the National Ignition Facility — which Bethe and Kidder support — will contribute to the eventual development of pure fusion weapons.

Colin Macilwain

Congressional critic backs amended deal on collider

[WASHINGTON] A modified agreement for US participation in the Large Hadron Collider project at the European Laboratory for Particle Physics (CERN) has been accepted by James Sensenbrenner (Republican, Wisconsin), chairman of the Science Committee in the House of Representatives, who had previously expressed reservations about the deal.

The modifications will now go to the CERN council in Geneva, Switzerland, for ratification. But Sensenbrenner warns that opposition remains strong in the Congress to a proposal that the United States should contribute to the collider's construction costs (see *Nature* 387, 3; 1997). He predicts that the proposal could face defeat unless the Clinton administration works harder to back it.

The modified deal was negotiated by CERN and the US Department of Energy in response to Sensenbrenner's concerns. It is believed to be similar in substance to the one initialled in February by Martha Krebs, assistant energy secretary, and Christopher Llewellyn Smith, director-general of CERN.

The modifications include a pledge in the introduction to the agreement that CERN member states will consider, in good faith, participation in future international particle physics projects. Cash amounts in the agreement are now prefixed with the phrase "not to exceed", and management arrangements are more clearly spelt out. For example, it is stated that the United States will be immediately informed if the machine is not going to

meet certain specified performance targets.

Officials on both sides of the Atlantic are quietly confident that the new deal will be accepted by the CERN council when it meets on 20 June. Federico Peña, the US energy secretary, said: "I believe we now have an amended and significantly strengthened agreement which, when ratified and signed, will better protect the interests of the United States." A spokesman for CERN said that Sensenbrenner "has done a good job" in clarifying specific points of the agreement.

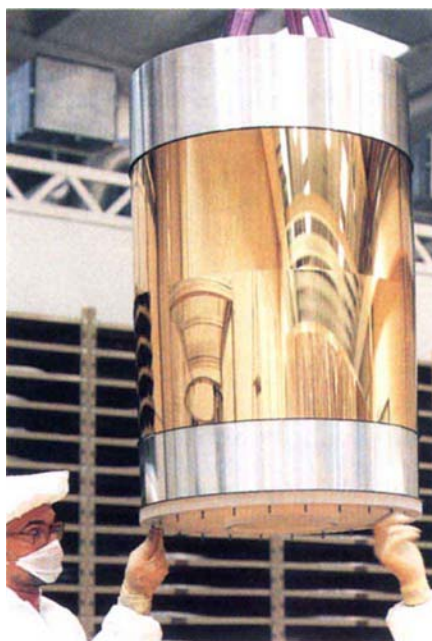
Sensenbrenner said that, if the deal is ratified by the CERN council, he would propose an amendment to next year's US budget to restore \$35 million for work related to the collider project which had previously been deleted by his committee.

But he said that he was "very disappointed that there has been no leadership from the energy department, the White House Office of Science and Technology Policy or the president" on justifying US participation in the project's construction. "Unless they hit a double, an amendment to restore that funding would be defeated." In baseball, a double is a good hit, rather like a four in cricket.

Funding for the collider project will now be considered by other committees in the Congress before they agree a 1998 budget in the autumn. Last week, nine US Nobel prizewinners in physics wrote to congressional leaders expressing support for participation in the project.

C.M.

Nest of mirrors promises X-ray relief



[MUNICH] Fifty-eight gold-plated X-ray mirrors nested together in shells, and separated by a distance of only 1–4 mm, form the telescopes on the European Space Agency's next cornerstone mission, an X-ray satellite known as XMM (see left). This unique technology gives XMM the largest X-ray collecting area of any currently planned X-ray mission.

XMM is also unique in carrying a sensitive optical telescope that will allow it to follow up X-ray observations in the optical range immediately. Scientists observing the X-ray 'afterglow' of short-acting gamma-ray bursts will no longer have to rely exclusively on ground-based telescopes to help identify their sources.

Tensions within the astrophysics community about access to ground-based telescopes were palpable after recent observations of gamma-ray bursts by the Italian satellite Beppo-SAX. XMM is due to be launched in August 1999.

UK redraws medical research funding

[LONDON] Britain's Medical Research Council (MRC) is introducing the biggest shake-up of its funding and peer review arrangements for university research for three decades.

According to MRC officials, the changes, whose details are being officially announced tomorrow (30 May), have been designed to encourage long-term, large-scale multidisciplinary research in the biomedical sciences. They also contain schemes intended to provide long-term finance for scientists who are starting out on their research careers, as well as a fund for short-term, high-risk, speculative research.

Some of the changes are already proving controversial, in particular the decision to phase out grants for stand-alone projects (see *Nature* 387, 330; 1997). The MRC will reduce its funding for such activities from the next academic session, which begins in October. The grants, which currently fund 170 projects worth around £10 million (US\$16 million) per year, will be phased out completely in 1998.

The MRC also plans to overhaul its research grant assessment system. Six existing grant application committees, each of them consisting of about 25 scientists, will be abolished. They will be replaced by a 200-member 'virtual' advisory board, primarily made up of existing committee members, but with a complement of new as well as younger scientists.

The changes will affect only the MRC's £300 million budget for research proposals initiated by scientists. Known as 'response mode' funding, this represents around half of the council's total research spend. The council's funding arrangements for its own research facilities will remain unchanged.

George Radda, the MRC's chief executive, says that the restructuring has been introduced because advances in medical

research are more likely to emerge from collaboration between high quality, interdisciplinary teams than from lone investigators or small research groups. "There is now more collaboration between basic and clinical scientists. They need to be supported and adequately resourced," he says. Radda says forging a closer association between universities was part of the thinking behind the restructuring.

Under the new arrangements, researchers will be able to apply for five types of response mode research grant (see below). Applications for centre grants, cooperative group grants and development grants will be assessed on their merits by members of the new 200-member advisory body.

Applications for career establishment grants will compete directly with one another, as will those for innovation grants. The former are intended, says Radda, to remove the uncertainty for new researchers who increasingly spend much of their early careers on a series of short-term contracts. Innovation grants are designed to protect speculative research.

The MRC, he says, will continue to provide support for "high quality" response mode applications in the form of 'programme grants'. Strategic programme grants made in response to specific initiatives will also continue as before, although in future they will also include funding for clinical trials under a subset fund known as 'trials grants'.

The proposals were drawn up by a small MRC working group after external consul-

tation. They were submitted for the first time to members of existing MRC grant committees at a discussion meeting held in London on 7 May.

The overall response appears to be positive. A member of one of the MRC's grant application committees sums up the views of many when he welcomes the plans as a "brave and constructive way forward".

"I'm particularly pleased that the MRC has maintained its commitment to response mode funding as opposed to going for more directed research," he says. "I also welcome the decision to ring-fence money for new researchers, so that they don't have to compete with experienced people."

This sense of relief, however, is tinged with some concern, particularly about the phasing out of funding for stand-alone projects. Some observers believe such a move will discriminate against high-quality researchers who are not necessarily linked to a large group or an élite institution. They also feel that it would reinforce the demarcation between 'research only' and 'teaching only' universities.

"The new proposals seem to consolidate the concept of 'centres of excellence'," said one member of an MRC grant committee. "British science has thrived from work done in small groups. We've had some great ideas from lone scientists. I'm a bit fearful that this type of 'string and sealing wax' science will disappear."

Others have privately expressed a degree of concern at the apparent lack of formal consultation with relevant and interested groups. The Committee of Vice-Chancellors and Principals of UK universities, for example, said it was unable to comment on the changes as it did not have enough time to consider the details.

A similar response came from the 100-member Association of Medical Research Charities (AMRC), which funds around £341 million of medical research in the United Kingdom per year. Diana Garnham, the AMRC's general secretary, says that the MRC restructuring plans seem to consolidate an existing trend in both the charity and public sector, as both the MRC and medical research charities have already cut back on stand-alone project funding.

Radda strongly denies the suggestion that the new schemes will lead to the concentration of research funds among a handful of universities. He points out that high-achieving scientists from smaller universities will still be funded, for example, if they choose to join the cooperative group grant scheme. On the question of consultation, Radda says "it was not possible to consult 100 universities and every medical research charity".

Ehsan Masood



Radda: seeking boost to long-term efforts.

New MRC 'response mode' funding schemes

Centre grants	Supporting research on a single university site. Five-yearly review. Sunset clause. Infrastructure provision.
Cooperative group grants	Supporting collaborative research within or between universities in single geographical area. Minimum three projects, one can be non-MRC-funded. Five-yearly review.
Development grants	Three-year, non-renewable grant for university to build up critical mass of research. 'Entry ticket' to cooperative grants.
Career establishment grants	Five-year, non-renewable support for research projects from newly appointed university scientists.
Innovation grants	One-year funding for high-risk, speculative research. Assessed from applicants' track record. Twenty awards worth up to £40,000 each.

Forged images lead to German inquiry

[MUNICH] In what appears to be Germany's first significant case of scientific fraud, two molecular biologists with rapidly growing reputations have been accused of fabricating data in papers published in leading journals.

Marion Brach, 37, a full professor at the University of Lübeck near Hamburg, is reported to have admitted falsifying results in four research papers while she was working in the laboratory of Friedhelm Herrmann at the Max Delbrück Centre for Molecular Medicine in Berlin in the early 1990s.

But Herrmann denies any knowledge that the data in the papers had been manipulated, even though he was a co-author on all four. Herrmann, 47, is one of Germany's leading gene therapy research scientists and a professor at the University of Ulm.

In the most brazen case, Brach, whose work concerns cytokines and multidrug-resistance genes in relation to cancer therapy, is said to have admitted that she had "mixed and matched" computerized images from unrelated experiments, based on unrelated methodologies, to forge a new set of experimental data.

Local and national committees have been set up to investigate the extent of the alleged fraud in the pair's voluminous publication record, and to try to determine whether Herrmann was aware of it.

Details of the affair were made public last week in the German news magazine *Focus*. Both scientists are continuing their academic work. But all grant money to their laboratories has been suspended until the affair is resolved. Herrmann's positions on advisory boards to the Deutsche Forschungsgemeinschaft, Germany's research council, have also been suspended.

Herrmann and Brach have shared what

has appeared to be a highly successful academic career. In the 1980s, both worked at Harvard University in the United States before returning to the University of Freiburg in Germany. In 1992 they moved to Berlin, where Herrmann was full professor at the Free University and a senior consultant at the university's Robert Rössle Cancer Centre.

Herrmann was also given laboratory space at Max Delbrück Centre, a national research centre set up in east Berlin after reunification, where he built up a research team of around 20 scientists. Brach was one of four group leaders in the team, a position that allowed her to gain the German qualification *Habilitation*, which is necessary to become a university professor.

In early 1996, Herrmann and Brach left Berlin for Ulm in west Germany, where Herrmann had better opportunities for his clinical and research activities.

Shortly afterwards Brach was offered a full professorship at the University of Lübeck. She is director of the university's new Institute of Molecular Medicine.

But the professional rise of both scientists was halted in March when investigations into the alleged fraud began. Researchers working with them in Berlin had for some time suspected that some data might have been fabricated, but had been reluctant to make a public complaint out of concern that Herrmann and Brach could hinder their careers.

Earlier this year, however, they took their evidence to a sympathetic intermediary, who alerted the deans of medicine at Ulm and



Herrmann: denies knowing of fraud.

Lübeck universities, as well as Detlev Ganten, director of the Max Delbrück Centre.

Guido Adler, dean of medicine at Ulm, asked Herrmann and Brach for written statements in response to the allegations. Brach is said to have eventually admitted falsifying data in four papers — two in *Blood*, one in *EMBO Journal* and one in *The Journal of Experimental Medicine* — but Herrmann denied involvement.

A local committee of investigation set up by Adler is likely to issue an interim conclusion next week about whether Herrmann was involved. The minister of science in the state of Baden-Württemberg is responsible for deciding what penalty to impose if Herrmann is proved to have been involved. Dismissal is a possibility, says Adler. "But it is hard to know what will happen because we have no experience of this in Germany." Adler has asked Herrmann to voluntarily give up his academic duties until the issue has been resolved.

Herrmann claims that when he was working in Berlin he left the running of the research projects in the hands of his four group leaders. Although he says that he read closely all the papers he co-authored, he did not recognize that data had been forged. "But neither did the journals' referees", says Herrmann.

He argues that the affair has been "hochgekocht" (overplayed), although he says he now accepts that data in at least one of the papers had clearly been fabricated. This is a paper in *The Journal of Experimental Medicine* about the control of transcription by the cytokine TNF (tumour necrosis factor) in human fibroblasts.

Among other deceptions (see figure), the paper presents as western blots, which are standard protein assays, old (non-radioactive) enzyme assays that had been stored as computer images. Smears of background radioactivity apparently went unnoticed by referees.

Ganten, who is a member of the national committee set up to investigate the affair, says he is astonished and disheartened that the papers appear to have passed through refereeing procedures without difficulty.

The national investigatory committee, made up of scientists and representatives of funding agencies, is studying other publications from the group to determine whether there are any further indications of fraud. It will also consider if grant money used for the fraudulent work should be repaid. It will report in the summer.

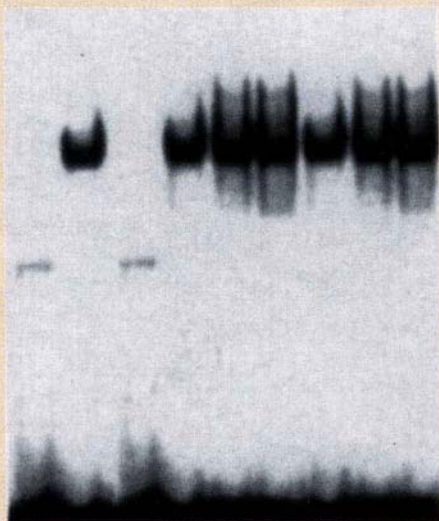
Most observers suspect that the deception was provoked by the increasing pressure on researchers in a 'publish-or-perish' environment, as well as the sudden opening of new opportunities in Germany in all areas of applied molecular biology.

Alison Abbott

Computer rolled three sets of data into one

One paper in which Brach admitted falsifying results is in *The Journal of Experimental Medicine* (181, 793; 1995). This figure, taken from the paper, shows an autoradiogram intended to indicate the intracellular location of transcription factors induced by the cytokine TNF. Yet it is composed of three unrelated sets of data. Also, the pattern of bands in the lanes indicates that one of the autoradiograms has been used twice in creating the figure.

Such manipulation of images is easy to achieve with the help of computers. John Tooze, editor of *EMBO Journal*, says that the ability to manipulate and enhance digitized images increases the opportunity for fraud, and therefore pressure on scientists reviewing potential journal articles to be more vigilant.



France rings changes in telecoms efforts

[PARIS] France is embarking on a major reorganization of its research in telecommunications, currently carried out mainly by the Centre National d'Études des Télécommunications (CNET), part of the state-owned company France Télécom.

The reforms include the creation of a national federation — the National Network for Telecommunications (RNRT) — intended to coordinate research carried out by France Télécom, which is soon to be privatized, and by public research organizations. There will also be an injection of FFfr1.3 billion (US\$230 million) in government funding over five years into a new research programme linking private companies and public research laboratories.

The reorganization has been prompted by the planned privatization. Until now, CNET has operated almost as a public research organization, serving not only France Télécom but also the entire telecommunications industry, including major manufacturers such as Alcatel-Alsthom. CNET's staff of more than 4,000 makes it the biggest telecommunications research centre in Europe.

The privatization presented a dilemma. Letting CNET stay with France Télécom would have left virtually no public research on telecommunications in France, denying other telecommunications companies a public research base. But transforming CNET into a separate public research body is seen as politically unacceptable.

François Fillon, the telecommunications minister, and François d'Aubert, secretary of state for research, have agreed on a compromise. CNET will remain with France Télécom, while public research will be boosted through a five-year programme intended to encourage research institutions — including the Centre National de la Recherche Scientifique (CNRS) and universities — to move into telecommu-

nications research.

CNET is also likely eventually to be reorganized. France Télécom does not make telecommunications components, and is therefore likely to shed its integrated-circuit laboratory at Meylan near Grenoble, and its opto-electronics laboratory at Bagneux, south of Paris. The two centres are already in limbo while the government explores solutions; these include linking Bagneux to Alcatel-Alsthom, and Meylan to SGS Thomson, according to one observer. Another possibility would be to create joint units between CNET and public research organizations.

The rebuilding of public research will in practice be carried out by bringing together public research organizations and CNET within the new RNRT. The RNRT will be managed by a steering committee chaired by Jean-Pierre Noblanc, the director of CEA-Industries, the technology transfer arm of the atomic energy commission, CEA. It will advise the government on research goals and propose projects within the new programme.

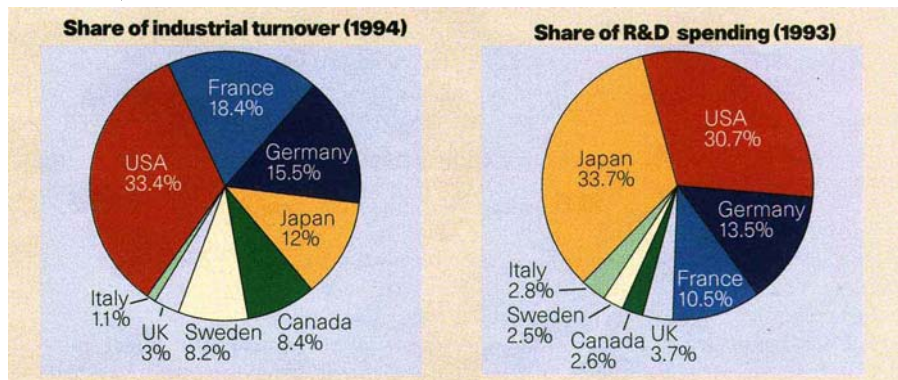
The committee's recommendation for projects will be translated into calls for proposals, issued by the ministry of telecommunica-

tions and ANVAR, the national agency for technology transfer, which will provide FFfr1 billion of new funding over five years for industrial research projects.

The science ministry will commit a further FFfr300 million for fundamental research. Projects must involve both a private company and a public research body, and grant recipients will be required at least to match the state contributions. As a result, the government estimates that total funding for the programme will amount to FFfr4 billion, a figure judged optimistic by many observers.

The role of the steering committee in proposing research projects will be critical to ensuring that the research funded by the programme corresponds to strategic needs, according to Gilles Kahn, scientific director of the national computing agency, INRIA, and co-author of the report on which the government's plans are based. Otherwise, there is a risk that researchers will simply relabel their existing research — on solid-state physics, for example — as telecommunications research to qualify for funding. Scientists are old hands at such tricks, says one observer.

Declan Butler & Robert Triendl



Crossed lines: Europe's share of telecommunications research lags that of its market presence

Britain will meet election commitment to rejoin Unesco

[PARIS] Britain's new Labour government has announced that it plans to meet its pre-election promise to rejoin the United Nations Educational, Scientific and Cultural Organization (Unesco). Britain left the organization in 1984 along with the United States, complaining of mismanagement and anti-Western bias.

The UK subscription of around £11 million annually will be a welcome addition to Unesco's annual budget of around \$250 million. But, according to one Unesco official, the greater significance of the United Kingdom's return will be to end what he describes as the "Anglo-Saxon cultural and linguistic absence" of the past decade. The biggest loss to Unesco from the US and UK withdrawal, he says, has been the absence of

the "two most important scientific and educational communities in the world".

A spokesperson for the UK Ministry of International Development says that the government is to rejoin because it is satisfied that Unesco has sufficiently reformed itself over the past 13 years, that it shares Unesco's ideals, and that it feels Britain can play a useful role in the organization.

The biggest change in Unesco science programmes over the past decade, claims one Unesco official, is the increased participation of scientific nongovernmental organizations. "People now look on Unesco as a respectable and responsive partner," he says. But critics still maintain that Unesco's science programmes remain weak (see *Nature* 385, 286; 1997).

President Bill Clinton has also indicated his desire to rejoin Unesco (see *Nature* 377, 569; 1995). But this has been prevented primarily because of the reluctance of the Republican majority in Congress to pay the \$65-million annual subscription.

A State Department official says that the United States will seriously consider rejoining if the administration and Congress reach agreement on the repayment of US debts to the United Nations in present negotiations. Putting US funding of the UN on "a sound financial footing" is a prerequisite for rejoining Unesco, he says. The agreement reached last week on the balancing of the US budget by 2002 has removed one obstacle to the negotiations.

Declan Butler

Company accused over lab data 'theft'

[SAN DIEGO] A small biotechnology company is being sued for allegedly making use of data that it is said to have stolen from a chemistry laboratory at the University of California at San Diego (UCSD).

Agouron Pharmaceuticals, Inc. of La Jolla is accused of taking data on the crystallization of an enzyme which its scientists used in a published article, and then in research aimed at developing an anticancer drug.

The lawsuit has been filed by Huguette Pelletier, now an assistant professor of biochemistry at Baylor College of Medicine in Houston, Texas. It could cause difficulties for Agouron, a publicly traded company whose shares have been riding high recently after it obtained federal approval for a new protease inhibitor as a potential anti-HIV drug.

The lawsuit centres on the activities of two Agouron researchers, Jay E. Davies II and his wife, Michele A. McTigue. Davies is the first author of the 1994 article, which describes the characteristics of the enzyme rat DNA polymerase β . He has also published articles on Agouron's protease-inhibitor project.

Both Davies and McTigue are former doctoral graduates of the UCSD chemistry programme, McTigue graduating in 1992. McTigue is being accused by Pelletier of using her personal access to facilities in the UCSD laboratory of Joseph Kraut to secure the polymerase β data secretly for her husband,

possibly by copying data stored on computer.

Pelletier also obtained her chemistry doctorate in Kraut's laboratory. Kraut, who is professor emeritus of chemistry and biochemistry, and is supporting Pelletier's allegation of McTigue's theft of enzyme data, was a co-founder of Agouron. But he cut his links with the company before the dispute arose because of a disagreement about strategy.

Davies and McTigue have denied Pelletier's claims, and Gary E. Friedman, an attorney with Agouron, denies that the company has acted improperly, saying that the allegations "aren't supported by the facts". (Agouron officials declined to be interviewed on the allegations.)

The allegation was first made in 1994, when Pelletier filed the lawsuit in San Diego Superior Court. After a three-year legal battle over her right to claim ownership of the data — which Agouron's attorney had disputed — Pelletier won a key legal decision from California's 4th District Court of Appeal last month, enabling her to sue the company.

The case will now go to trial in the Superior Court, where it is being assigned a new judge because the judge who had originally ruled erroneously in favour of Agouron has since resigned following a drunken-driving conviction.

The Agouron article in question — the first on crystallization of rat DNA polymerase

β — was published in the journal *Cell* in March 1994 (76, 1123–1133; 1994). Pelletier and Kraut have since written a number of letters to the editor of *Cell*, Benjamin Lewin, seeking assistance in resolving what they call several "scientific discrepancies" and "misleading" statements in the Agouron paper.

Pelletier has also urged *Cell* to persuade the Agouron team to file all the back-up data for the crystallography — coordinates and structure factors — with the Protein Data Bank at Brookhaven National Laboratory in New York state.

After the first letter to *Cell* in September 1994, one set of coordinates was filed with the data bank. But records show that no corresponding structure factors were filed, and coordinates on other crystallography in the paper have yet to be lodged at the data bank.

Pelletier claims that the Agouron research team has been dragging its feet on filing the data because publication might reveal data that were generated by her.

Pelletier's research on polymerase β was eventually published in October 1996 in *Biochemistry* (35, 12742–12761; 1996). She blames the delay on Agouron, in particular its publication of the paper containing what she claims to be her data. Discrepancies between her paper and that published by Agouron have already prompted considerable debate among crystallographers.

Rex Dalton

Tighter evaluation urged for Japan's public research bodies

[TOKYO] A report aiming to formulate guiding principles by which all publicly funded research in Japan — including that carried out in industry — should be evaluated was released last week by an advisory body attached to the prime minister's office.

The report, by a subcommittee of the Council for Science and Technology, concedes that the present state of research evaluation in Japan is "very unsatisfactory". It says that evaluation is central to the building up of a new research system, and that the results of evaluation exercises should be fed directly into future funding decisions.

But it does not make any suggestions about how this should be achieved. And the choice of evaluation practices and the procedures by which they are to be implemented is left to individual research institutions and the funding bodies that oversee them.

Guidelines for assessing research in national institutes were first issued by the council in 1986. But a recent review showed that fewer than one third of Japan's public research centres had put the

recommendations into practice.

Many observers feel that the report's recommendations are largely token measures intended to ensure that funding for science is maintained at a time when there are growing political pressures to reform the country's national budget. They also criticize its lack of consideration of how to assess research policies.

"What we need in Japan is a broad discussion on the practicalities of research funding," says Shinichi Kobayashi, a science policy analyst at the University of Electro-Communications in Chofu, near Tokyo. "Policy and programme evaluation should come first."

Kobayashi says that many research funding bodies do not have the means to carry out their work properly, citing the Ministry of Education's general university grants scheme as an example. Funds allocated through the scheme have more than trebled in recent years. But roughly 100,000 applications a year continue to be processed by only seven administrative staff.

Masahiro Kawasaki, vice-president of the Japan Science and Technology Corporation, a funding body attached to the Science and

Technology Agency, says that the report concerns mainly the "basic methodological aspects" of evaluation. "Policy considerations will have to follow," he says.

Much depends on how individual agencies, research institutions and universities implement the new guidelines. The Ministry of International Trade and Industry's Agency for Industrial Science and Technology (AIST) has already announced plans to set up a division for research evaluation in July. But similar initiatives at other ministries are said to have been blocked by the finance ministry.

According to an official at AIST, all future proposals for research projects will have to undergo an initial peer review. Accepted projects will be reviewed at least once during their lifetimes, and all projects will be limited to five years. Until recently, research consortia financed by AIST were evaluated only after they had finished their work, and against goals defined at the outset. But, as the final evaluation was usually done by those responsible for the project, the results "were often manipulated to correspond to initial project goals", says an official at the agency.

Robert Triendl

Ethics of private panels comes under scrutiny

[WASHINGTON] Private ethics boards set up to review experiments carried out mainly by pharmaceutical companies and medical products manufacturers came under vigorous attack at a congressional hearing early this month for allegedly corrupting the US system of human subjects protection. But they are being equally vigorously defended.

A hearing of the House of Representatives Government Reform Committee's human resources subcommittee was told by some witnesses that private Institutional Review Boards (IRBs) were commercially motivated, and therefore unduly likely to approve undeserving experimental protocols.

They were also charged with opening the door to 'IRB shopping', when a researcher turned down by one IRB will take it to another prepared to approve it. (Academically affiliated researchers cannot engage in such 'shopping', as they are required to use their own institution's IRB.)

But others, including an official from the Food and Drug Administration (FDA), which is responsible for monitoring the activities of most private IRBs, challenge these claims. They say that in practice private IRBs differ little from the local, not-for-profit IRBs set up by research hospitals and universities to ensure that research is ethical and protects human subjects.

The private IRBs are not attached to any particular institution. They are used most frequently in late-stage clinical trials by the producers of drugs and medical devices, and the private physicians they employ.

Some of these boards also monitor other research, ranging from the development of *in utero* genetic tests to studies of dose size for drugs at earlier stages of development.

There are fewer than half a dozen studies funded by the National Institutes of Health which employ a private IRB. One of these is developing diagnostic and treatment-monitoring software for psychiatry.

There are thought to be up to 12 large private IRBs operating in the United States, with an unknown number of smaller ones. Their founders range from retired physicians to researchers originally from academic institutions or industry. Their members include nurses, clergy, scientists and doctors.

Critics at the hearing included Benjamin Wilfond, a paediatrician at the University of Arizona. He argues that "in the commercial sector, there is at least a greater potential and incentive for approving protocols that may be questionable, that may have a concern about the risks". Approvals from private IRBs, he says, keep customers satisfied and coming back.

Wilfond said he recently discovered a private doctor in Tucson carrying out a trial financed by a drug company of a new asthma drug in children, with approval from a private, out-of-state IRB. The University of Arizona's IRB had found the trial unethical, because it required stopping children taking effective drugs.

Wilfond believes that "there needs to be more universal regulation of research so that consistent standards apply to both private sector and academic sector". Christopher Shays (Republican, Connecticut), the subcommittee chairman, said he found it "amazing" that private IRBs were not subject to certification. But some argue that such charges are alarmist. Robert J. Levine, for example, a professor at the Yale University School of Medicine who chairs Yale's IRB, called it



"ridiculous" to present IRB shopping as more than an occasional, if unfortunate, event in a system that worked well overall.

Levine said that researchers heard about IRB shopping "about as often as we hear about the birth of Siamese twins". Others argued that private IRBs are no more vulnerable to financial incentives for approving experiments than those at universities, which depend heavily on grants brought in by government-funded researchers.

The FDA — which audits both types of IRBs — says that it also lacks proof of major problems with private IRBs. Paul Goebel, associate director for human subject protection in FDA's Office of Health Affairs, says he is not convinced that private IRBs "as a category" do a worse job than the academic IRBs, with a wide variation in the competence with which each carried out their tasks.

Whatever their quality, the purview of private IRBs is likely to grow with the increasing privatization of research support. "There's a brave new world looming and I'm not sure the regulatory system was constructed with that world in mind," said Arthur Caplan, director of the University of Pennsylvania's Center for Bioethics.

The only private research at present required by law to receive IRB approval is that done by makers of drugs, medical devices and other FDA-regulated products. These companies often employ private physicians to carry out late-stage trials. Those without institutional affiliations therefore have to find private IRBs. By contrast, privately funded researchers at universities are generally required to submit their proposals to their institution's IRBs.

Under a bill introduced by Senator John Glenn (Democrat, Ohio), approval by an IRB would become mandatory for private-sector researchers.

The Human Research Subject Protections Act of 1997 was introduced in the Senate in January, but has just three co-sponsors. The act is opposed by the drug industry, partly because it would impose criminal penalties on violators.

Meredith Wadman

Indifferent performance on privacy protection

[WASHINGTON] Institutional Review Boards (IRBs) vary in willingness and ability to protect the privacy of human subjects in medical research, according to a report commissioned by Donna Shalala, the US Health and Human Services Secretary, which was released last week.

The report, *Privacy and Health Research*, by William Lowrance, a health policy consultant, says there is "no doubt" that IRBs enhance the protection of research

subjects. "But it is less clear that IRBs have been attending as vigorously to privacy risks as they have to physical and emotional risks," it says, suggesting that "whether they are able and willing to do so should be assessed".

The focus of the report is on balancing individual privacy protection with medical research. It concludes that, for much research outside formal clinical trials — such as epidemiological reviews and secondary studies in

databases — "notice is not routinely given nor explicit [informed] consent sought".

Lawrence Gostin, a professor of law and public health at Georgetown University Law Center in Washington DC, and an expert on medical privacy issues, says the rules governing IRBs are vague on "it's commonly thought that government research regulations have a vigorous privacy protection that the IRB has to follow," he says. "They don't."

M.W.

Human Frontier Science Programme seeks more funds

[MUNICH] The Human Frontier Science Programme, the Japanese-inspired project that funds intercontinental collaboration in basic biological research, will continue for at least a further five years.

The programme's intergovernmental conference in Washington last week agreed that the project should seek to increase its financial resources to meet growing demands. At present, only 10 per cent of applications for grants and 19 per cent for fellowships are approved.

Around 80 per cent of the programme's support comes from Japan. But in 1992 it was agreed that the eight other Western members should between them match the US\$30-million annual contribution paid by Japan. Last week's conference recommended that each member should increase its subscription to a level proportional to its gross national product over a five-year period.

The meeting also recommended that any increased funding be spent on expanding the grant programme rather than the fellowship programme. Most members said they would try to increase their contributions, but no commitments have yet been made.

Trial failure sends biotech shares plummeting

[LONDON] Shares in the UK biotechnology company Celltech lost almost 50 per cent of their value last week following the company's decision to abandon a treatment for septic shock after disappointing trial results. The value of the shares fell from 630 pence (US\$ 10.25) to 332 pence at news that the drug, BAYX-1351, which was being produced in association with the pharmaceutical company Bayer, had failed in a trial of 2,000 people in the United States.

The news prompted other UK biotechnology shares to fall. Some of the worst hit include Cantab Pharmaceuticals (down 47.5 pence to 917 pence), and PPL Therapeutics, the company involved in cloning Dolly the sheep (down 15 pence to 407.5 pence).

Royal Society sets out education agenda

[LONDON] Britain's Royal Society has proposed the creation of a Teaching and Learning Council to respond to the changing needs of an innovative and quality education system for the next century. Speaking in London on 19 May, Sir John Horlock, the society's treasurer and vice-president, expressed particular concern about

suggestions that there may be a further transfer of funds from the higher education funding councils to the research councils.

Horlock said that it was important to maintain two distinct funding systems with complementary functions operating under the dual-support system. Other issues that have been raised by the society in its submission to the new Labour government setting out its views on higher education are the need to preserve a diversity of support and to enhance assessment and selectivity in research funding.

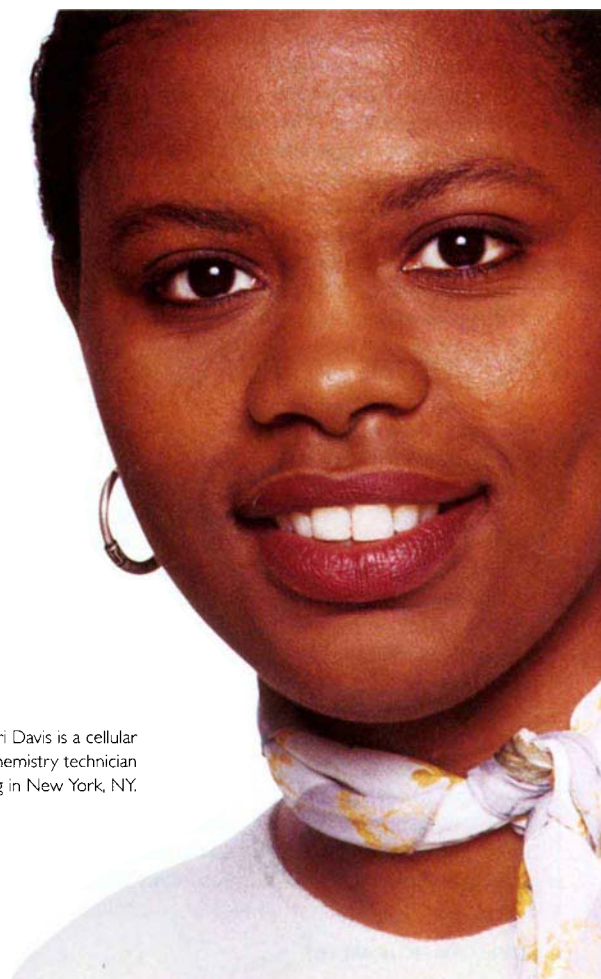
Chinese researchers scoop German grants

[MUNICH] Chinese researchers have, for the first time, headed the list of fellowships granted by the Alexander von Humboldt Foundation, Germany's main research grant-giving body for young foreign scientists. The foundation, established in 1953, supports 'guest researchers' for one year or more in Germany. Eighty per cent of Humboldt research fellows are hosted by universities.

According to the foundation's new annual report, 1,427 scientists from 93 countries were granted research fellowships in 1996. Less than half came from Europe. China (145) is followed by the United States (142), the Russian Federation (140) and India (103).

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Terri Davis is a cellular
biochemistry technician
working in New York, NY.



Nuclear agency seeks new inspection powers

[LONDON] The board of governors of the International Atomic Energy Agency, based in Vienna, has approved plans to strengthen the agency's powers to detect clandestine nuclear activity from undeclared nuclear weapons states.

The plans, which take the form of an additional protocol to an existing agency agreement, will now be put to 186 countries for ratification. If accepted, the new protocol will provide the agency's inspectors with the powers to seek information from countries about nuclear fuel cycles and nuclear exports.

Boost for equipment funding scheme

[LONDON] Britain's Wellcome Trust is to continue funding a six-year-old scheme that pays for research equipment costing more than £60,000 (US\$98,000). But the trust is to merge part of its grant-giving operation with a similar exercise jointly managed by two research councils and the Higher Education Funding Council for England.

The trust will continue to process equipment funding applications and award grants. But copies of some applications will also be passed to the funding councils for

consideration under their Joint Research Equipment Initiative. The Wellcome Trust will be reimbursed 50 per cent of the equipment costs for those proposals that are successful under the JREI scheme. The money will be used to enhance the trust's equipment budget.

Scientist named as lieutenant governor

[SYDNEY] Adrienne Clarke, professor of botany at the University of Melbourne, has been appointed Lieutenant Governor of the



State of Victoria, the first time this position has been occupied by an Australian scientist. The post is usually held by a retired judge or general. Clarke, who served on the board of the Commonwealth

Scientific and Industrial Research Organisation for ten years, five years as chairman, will combine her new part-time post with her academic responsibilities.

An unusual aspect of her appointment is that she has been charged with promoting science rather than acting ceremonially as deputy for the Governor (a full-time, non-elected vice-regent in the state for the Queen of Australia and Great Britain).

Japan set to miss target to cut carbon emissions

[TOKYO] Japan's carbon dioxide emissions increased by 0.5 per cent in 1995 to a record 332 million tonnes, making it increasing unlikely that Japan will realize a commitment made with other leading industrialized nations at the Rio summit in 1992 to reduce emissions to 1990 levels by 2000.

Some of this increase, which has yet to be officially confirmed, is due to population growth. Total emissions increased by 8.3 per cent between 1990 and 1995 in Japan. But the per capita emission rate increased from 2.59 tonnes in 1990 to 2.74 tonnes in 1994.

Tax wrangle drives Soros from Belorussia

[MOSCOW] The Belorussian Soros Foundation, set up by the Hungarian-born financier George Soros, has quit Belorussia after US\$1.78 million was taken from its bank accounts by the government's tax office, despite tax exemptions that had been guaranteed by the government two years ago.

The foundation has spent \$13 million supporting scientific and cultural programmes. But a state committee challenged some of its projects, including ecological archives relating to the Chernobyl accident (see *Nature* 387, 117; 1997).

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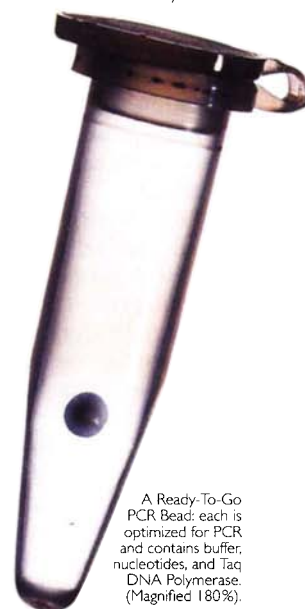
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Problems of postgraduates and PhDs

Sir — The lack of jobs that Alan Hale bemoans (*Nature* **386**, 530; 1997) should interest all of us. The problem, I suspect, is that there are too many postdocs, while academic institutions scramble for yet more postgraduates to sustain their research output cheaply. The supply of PhDs, therefore, is decoupled from the demand for postdocs. As with theatres that sell more tickets than there are seats available, the crowd of dissatisfied customers who cannot secure a position becomes a growing embarrassment. The solution (and I use the term loosely) that most universities opt for is to encourage graduates into alternative careers and quell the crowd each year by blaming market forces or governments. But injecting more money into science, while welcome, will bring only temporary relief. The problem will persist until universities take responsible action and base their postgraduate intake for a given field of research on the employment of their previous year's PhD graduates in that field. This will go some way to restore the balance between supply and demand.

In present circumstances one might expect a vigorous and beneficial competition whereby the best get best-placed. But a consequence of any glut is

devaluation, and in the case of postdocs this has meant a loss of academic independence. So the research performance of young scientists depends as much on their laboratory and group leader as on their own potential. If we are to equate the performance of young scientists with their abilities, postdocs should have more opportunities to work and publish as independent scientists.

To implement these changes, there must be a profound shift in attitude, with scientists, research institutes, granting bodies and governments unilaterally taking responsible action. The alternative is a deterioration of the current situation from which we all stand to lose.

Gerard Vassiliou

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Sir — Postgraduate students in the United Kingdom are shackled to a feudal system. The supervisor has total responsibility and control over research training. Regardless of contribution, the supervisor has the right to

claim one's work for him or herself, as is manifest in any patents and publications resulting from the student's efforts. How many professors with a DSc can claim that it is due to their efforts alone rather than to the exploitation of student vassalage?

I am awaiting the results of the umpteenth round of the grievance procedure at an English university (initiated in December 1995). My complaints range from financial duplicity to the deliberate withholding of materials, equipment and data required to complete a PhD. My problems have arisen through conflict with a supervisor, which is not uncommon. The problems could be more easily dealt with if research students were registered directly with the research institute and not with a particular individual, whereupon the term 'supervisor' becomes merely titular. And it would be less tempting to any mercenary supervisors if all the 'intellectual property' used and developed by the student was the property of the student and the institute alone.

James McComb

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Isle of Wight PO30 1ET, UK*

Compatibility of Islam and science

Sir — The surprise that greeted the survey of religious belief in the scientific community (*Nature* **386**, 435; 1997) makes me wonder whether more scientists would believe in God if the predominant religious tradition of their upbringing had been not Judaeo-Christian but Islamic. While Western science developed in antagonism to the Church, Islam's scientific tradition, recognized as initiating Europe's scientific renaissance, grew not in conflict to the religion but within it.

The absence of a dichotomy between science and religion in Islam stems, I believe, from the Koran's active encouragement of scientific enquiry. This scriptural endorsement of science is supported by the complete lack of statements in the Koran that need to be explained away because they contradict established facts about nature. In fact, Koranic descriptions, often surprisingly detailed, of the origin of the Universe, the motion of the planets, the developmental progression of the human embryo, the existence of sexual dimorphism in plants

and the structure of mountains have been confirmed by scientific discovery centuries later.

To the Muslim scientist, the surprise is not that so many scientists believe in God but that so many do not. The Koran states (21:30): "I have created from water every living thing. Will they not believe?"

Nadeem Ali

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Sir — Assuming that the forthcoming test of the efficacy of prayer in the treatment of heart-surgery patients (*Nature* **386**, 639; 1997) will be done with proper double-blind protocols, there remains the question of whether the experimenters are willing to accept results that are not in line with their faith.

What if the test shows no effect of prayer, or that the prayed-for people actually do a little worse? Will they still publicize the results, and without excuses?

Edgar Pearlstein

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No PICES advice

Sir — There are some factual errors in the Briefing on fishery science (*Nature* **386**, 106; 1997). It is said that PICES (the North Pacific Marine Science Organization) is one of two bodies that exist to provide governments with advice on fisheries science (the other being ICES, the International Council for the Exploration of the Sea).

PICES, which held its first meetings in 1992 (not 1994), was set up to promote and coordinate marine scientific research in the northern North Pacific. Until now, its members (Canada, China, Japan, Korea, Russia and the United States) have not agreed to a PICES role in providing advice on fishery questions.

Elsewhere in the article, it is said that ICES and PICES have long recognized that there are alternatives to giving advice on catch limits. However reasonable that position may be, it is not one that PICES has addressed.

Warren S. Wooster

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Cultivating a cure for blindness

Stuart Hodson

Damaged corneas can often be repaired using donor grafts, but if the damage is too great the graft will be rejected. This may change with the development of a method to inhibit rejection which uses cultivated cells.

The human cornea has a special property known as immune privilege, which allows tissue grafts from donors to be carried out without the usual problems of immune rejection. However, immune privilege is lost at the perimeter of the cornea — the limbus of the eye — where the transparent corneal stroma meets the opaque sclera (Fig. 1). So, if too large a corneal button is grafted and it approaches the limbus of the recipient, the graft is usually rejected. Similarly, the prognosis is not good if the recipient's cornea is so badly damaged that there is no sign of healthy corneal tissue.

A solution to these problems may now be at hand with the publication of a report by Pellegrini *et al.*¹ in *The Lancet*. They have extended immune privilege in an unexpected way with the aid of human stem cells, and their results promise a wider use of corneal transplantation in what are currently thought to be relatively untreatable conditions.

The outer layer of the cornea comprises the regularly dividing and sloughing corneal epithelial cells. Studies into the origin of these cells showed that they are not derived by self-perpetuation — instead they come from stem cells in the limbal epithelium^{2,3}, which occupies an annulus (ring) about 1.5 mm wide between the corneal epithelium and the conjunctival epithelium (Fig. 1). The transition from corneal to limbal epithelial cells is quite abrupt, but whereas the corneal

epithelium can mould a smooth apical and basal surface, the limbal epithelial stem cells do not form such a smooth surface.

If the corneal epithelium is lost it can be functionally regenerated by the limbal stem cells. But if both the corneal and limbal epithelia are lost, the corneal surface is recolonized by the other neighbour of the limbal epithelium — the conjunctival epithelium³⁻⁵. After a cornea has been grafted into a recipient, its outer surface will eventually be covered by a host-derived epithelium that migrates in from the limbus. So, if the recipient has limbal epithelial cells, a normal corneal epithelium is generated. But if the eye has been severely damaged and the recipient's limbal epithelium is missing, the donor cornea will be covered, by cell migration, with cells from the conjunctival epithelium. Conjunctival epithelia and corneal/limbal epithelia represent two different cell lines⁶, and the conjunctival epithelium cannot form smooth apical and basal surfaces if it is allowed to colonize the cornea. The result is a loss of visual acuity by scarring of the stroma, as well as severe discomfort.

Pellegrini *et al.*¹ now describe a method for using limbal stem cells to advantage in corneal epithelial grafts. Two patients, who each had one severely damaged cornea that was covered with conjunctival epithelium, were willing subjects after conventional grafting had failed. Limbal epithelial cells

were taken in a 1-mm² biopsy from the good eye, then they were cultured and, on second passage, they formed a tightly packed and communicating (confluent) monolayer of cells. For grafting, the conjunctival epithelium was completely removed from the cornea and limbus of the recipient eye, and replaced with a slightly larger monolayer of cultured limbal epithelium. The eyes were then covered with therapeutic soft contact lenses, and tightly patched for several days.

The results after the limbal epithelial graft were very promising. The grafted epithelium was stable, transparent, multi-layered and smooth. One of the patients had suffered an alkali burn to his left eye ten years earlier, and had undergone three previous unsuccessful corneal grafts. Before the treatment he had continual severe corneal vascularization (development of blood vessels) and persistent ulceration, and the eye was painful and blind. But after the conjunctival epithelium that was covering his cornea was replaced with cultured limbal epithelium, a stable and transparent corneal epithelium was reconstituted, and there were no further problems. His vision improved so much that he could count fingers from one metre away. However, because of his previous experiences he refused the next logical step, which was to check whether his cornea had recovered its immune privilege and could now take a full-thickness graft.

The other patient, whose epithelial graft using the new method had also been a success, agreed to a second operation of a full-thickness graft. The result was excellent — the eye that had been blind for many years, and had been perforated after an earlier unsuccessful graft, was restored to a visual acuity of a high enough standard to allow him to drive a vehicle safely using the restored eye alone. His previously untreatable blindness was cured after 29 years.

The clinical results are surprising and encouraging. Clearly, much of the cornea's immune privilege is vested in the limbal/corneal epithelium, and other epithelial cell lines seem to be unable to inherit this privilege. It would be nice to see the development of limbal epithelial allografts (for which tissue matching would be necessary), to find out whether they could also extend the immune privilege of the cornea. It could then be possible to restore full vision to millions of totally blind people through limbal epithelial primary grafts.

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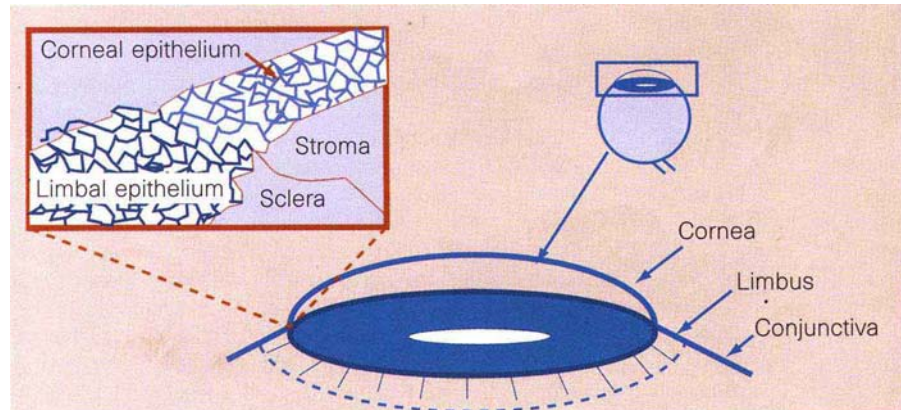


Figure 1 Donor grafts can often be used to repair damaged corneas, as the donor's corneal epithelium can be replaced by stem cells of the limbal epithelium. If the recipient's cornea is too badly damaged and the neighbouring limbal epithelial cells are also lost, conjunctival epithelium will extend to replace the corneal epithelium and the grafted cornea's 'immune privilege' will be compromised. Pellegrini *et al.*¹ have now developed a method to inhibit rejection, by restoring the corneal surface using sheets of cultivated host limbal epithelium as a primary graft prior to full-thickness grafting.

A sense-*abl* response?

Lamorna Brown and Nicola McCarthy

In the old days, radiation biology seemed a fairly straightforward affair — zap a cell and it stopped and died because it could no longer replicate its DNA. Nowadays, the world is both more curious and more complicated. The DNA damage inflicted by radiation (or by genotoxic drugs) is detected by a dedicated DNA-damage-sensing apparatus, which then triggers a number of possible effector pathways that can lead to DNA repair, growth arrest or programmed cell death (apoptosis). Which fate prevails seems to depend on the severity of the damage, the type of cell affected and the cellular environment, presumably reflecting the potential risk that the affected cell presents to the organism of which it is a part (see box).

The study of patients with inherited predispositions to cancer has provided many insights into the nature of the DNA-damage response in man. More clues now emerge from two studies by Baskaran *et al.*¹ and Shafman *et al.*² on pages 516 and 520, respectively, of this issue. They have found a direct interaction between two proteins that are involved in the cellular response to DNA damage.

Because cancers arise by somatic muta-

tion, genetic predispositions must reflect faults either in the machineries that sense DNA damage, or in those that repair or delete damaged cells. Ataxia telangiectasia (AT) is a rare recessive disorder that is associated with a plethora of phenotypes, including unsteady posture (ataxia), dilated blood vessels (telangiectasia), neuronal degeneration, immune dysfunction and an increased incidence of cancer³. Up to 1 in 100 people are heterozygous for the gene that is mutated in AT (*ATM*), and these people have a significantly increased risk of cancer compared with the general population.

Although it is unclear how all of the pathologies associated with AT are linked to the underlying genetic defect, several aspects of the AT phenotype suggest a fault in the cellular response to DNA damage. The *ATM* gene has been cloned⁴, and it encodes a nuclear phosphoprotein (*M_r* 350,000), the carboxy terminus of which shares homology with the catalytic domains of members of the eponymous phosphatidylinositol 3-kinase family. Members of this family have been implicated in cell signalling, cell-cycle checkpoint regulation, meiotic recombination, monitoring of telomere length, and

DNA-damage responses. However, AT cells fail to arrest in the G1 or G2 stages of the cell cycle in response to ionizing radiation; a defect that is most consistent with a role for ATM in either sensing, or responding to, DNA damage. Moreover, ATM shares homology with the catalytic subunit of DNA-dependent protein kinase, which is also implicated in the response to DNA damage⁵.

DNA damage in mammalian cells triggers three pathways — DNA repair, cell-cycle arrest and apoptosis. Each comprises a distinct set of effectors that implement processes which, once triggered, are largely independent of the other two pathways. The papers by Baskaran *et al.*¹ and Shafman *et al.*² now go some way to addressing how these three different pathways are coordinated and how the damage is recognized, by establishing a link between ATM and c-Abl, a protein kinase that has already been implicated in the growth-arrest response to DNA damage.

Shafman and colleagues show that, both *in vivo* and *in vitro*, ATM binds to the SH3 domain of c-Abl through a central proline-rich domain. When cells are treated with ionizing radiation, c-Abl is activated, but only in the presence of functional ATM: naturally occurring *ATM* mutant cells derived from AT patients do not interact with c-Abl. In wild-type cells, the association between ATM and c-Abl seems to be constitutive, and is not enhanced by treatment with ionizing radiation. However, ATM only activates the c-Abl tyrosine kinase activity following DNA damage. So although ATM seems to be necessary for activation of c-Abl, some signal is required to trigger activation after DNA damage.

Baskaran and colleagues¹ dissect the potential of the ATM kinase domain to activate c-Abl. They show that mouse embryo fibroblasts (MEFs) derived from the *ATM* knockout mouse, which has a similar phenotype to that of human AT (ref. 6), are defective for radiation-induced activation of c-Abl. Moreover, expression of the ATM kinase domain alone is enough to restore the activity of c-Abl, and the authors show that, within the tyrosine kinase domain of c-Abl, serine-465 is the critical target for ATM activation.

Surprisingly, the ATM kinase domain can activate c-Abl in MEFs, even in the absence of DNA damage. Again, this implies that there is some other tier of control which ensures that c-Abl is activated by ATM only in response to DNA damage. Such regulation might involve other portions of the large ATM molecule, but although it is possible that ATM itself senses DNA damage, it is equally plausible that another protein senses the damage and then activates ATM.

Baskaran *et al.* detect no apparent cell-cycle checkpoint defects in irradiated

A question of DNA repair

For unicellular organisms, the response to DNA damage is simple — the damage is contained by arresting the replicative machinery, then DNA-repair programmes are activated to fix it. Any mutations that are acquired in the process are merely the spice of unicellular genetic diversity. But metazoan cells must delegate their autonomy to the needs of the multicellular organism of which they are a part: if a mutation occurs in the wrong genes, the rogue cell and its progeny could stir up neoplastic Armageddon. So DNA repair is probably reserved for mending minor DNA lesions, rather than for mounting rescue operations to

save just one cell. In metazoans it is almost always 'safer' to delete the affected cell as opposed to trying to rebuild it, particularly as most somatic mutations occur in proliferating tissues, wherein the replacement of cells is trivial.

In fact, just a single double-stranded DNA break is enough to trigger — through p53 — either permanent growth arrest or apoptosis. Such sensitivity to the activation of cell-deletion pathways following DNA damage raises complicated questions concerning the fates of cells that can no longer sense DNA damage effectively. Would such cells be resistant to ionizing radiation, because they

fail to activate their apoptotic programme, or sensitive, because they fail to mount any repair response and so accumulate unrepaired DNA damage? Would the organism harbouring such cells be more at risk from cancer, because its somatic cells fail to arrest or die following DNA damage, so they survive with their mutated DNA? Or would such an organism be less prone to cancer because its cells fail to repair damaged DNA? The answers to these critical questions require further insight into how the sensing and effector responses of DNA damage are integrated and configured.

Gerard Evan

abl^{-/-} null MEFs, contradicting an earlier observation which indicated that c-Abl is involved in DNA-damage-induced G1 arrest. If true, the new result may mean that the ATM/c-Abl pathway is not responsible for the cell-cycle-arrest arm of the response to DNA damage. Indeed, Baskaran *et al.* believe that the ATM/c-Abl response modulates the expression of target genes by phosphorylating RNA polymerase II (which is a known substrate of the c-Abl kinase⁷), and/or by triggering stress kinase pathways such as the SAPK/JNK pathway⁸ (Fig. 1), as suggested by Shafman *et al.*². But it is possible that MEFs from *abl*^{-/-} mice have simply adapted to absence of c-Abl during development, and 'learnt' to recruit other effector pathways to ensure growth arrest following DNA damage.

How might this damage-sensing apparatus be connected to the effectors that mediate the cellular responses to DNA damage — repair, growth arrest and apoptosis? An obvious intermediary is p53 — not only is this protein pivotal in DNA-damage responses, but its damage-dependent activation is compromised in AT cells⁶. Moreover, DNA damage triggers a direct physical association between c-Abl and p53 (ref. 9). However, Baskaran *et al.* find that stabilization of p53, which is a characteristic of the p53 response to DNA damage, does not seem to require c-Abl; nor, indeed, is p53 needed for activation of c-Abl following DNA damage. Presumably, c-Abl and p53 are distinct targets of ATM, although their subsequent interaction may coordinate the various effector pathways in the damage response.

Any tidy picture of the activity of ATM and c-Abl is further confounded by the other interesting targets of each protein. For ATM, these include the transcription factor NF- κ B,

Archaeology

Prehistoric monsters

In France, the bohemian tradition goes back a long way. Palaeolithic cave artists of the Lot valley in southwestern France not only experimented with surrealism, they may also have found inspiration in hallucinogenic drugs. So say Michel Lorblanchet and Ann Sieveking, in an analysis of engravings in the innermost room (IV) of the cave of Pergouset (*Camb. Archaeol. J.* 7, 37–56; 1997).

Room IV is hard to reach; the passage is narrow, mud-choked and steep. Once there, the explorer faces a sloping ceiling on which a fantastic bestiary is engraved. Part of it is transcribed here, showing a long-necked creature with a horse-like head, and next to it a "monstrous head" that is harder to categorize. Elsewhere there are both less and more peculiar creatures, including one that vaguely resembles a fox's head on two narrow legs.

Why did the engraver suddenly abandon the conventional art of the time (probably the Magdalenian period, around 12,000 to 15,000 years ago), which fills the first three rooms of the cave? Lorblanchet and Sieveking believe that it is a sign of an "altered state of consciousness", perhaps brought on by a hallucinogen — most probably the Fly Agaric mushroom. But they allow that it may simply indicate "a



propensity to fantasize".

In any case, the location was probably important. There is evidence that this room was not regularly visited, so the artist may have been uninhibited by the prospect of criticism. And the shape of the room may have added some magical significance, creating "an illusion that these imaginary creatures emerge from the darkness of the gallery beyond, or the floor below".

Stephen Battersby

which is implicated in various stress and apoptotic processes, and telomeric proteins. Telomeres shorten with each progressive cell cycle, and they trigger cell senescence once they reach a critical minimum length¹⁰. AT

cells often have short telomeres, and fibroblasts from ATM-deficient mice senesce rapidly in culture, supporting a telomeric role for ATM (ref. 6). c-Abl, on the other hand, interacts with the retinoblastoma protein, which is a key regulator of the G1/S cell-cycle transition¹¹. The significance of this is unclear, given that the growth-arrest response is normal in c-Abl-deficient cells. But what Baskaran *et al.*¹ and Shafman *et al.*² have done is to establish a connection between ATM, the detection of radiation-induced DNA damage, and the activation of c-Abl. Clues as to how this damage-sensing apparatus interacts with other proteins should now follow on from this work. □

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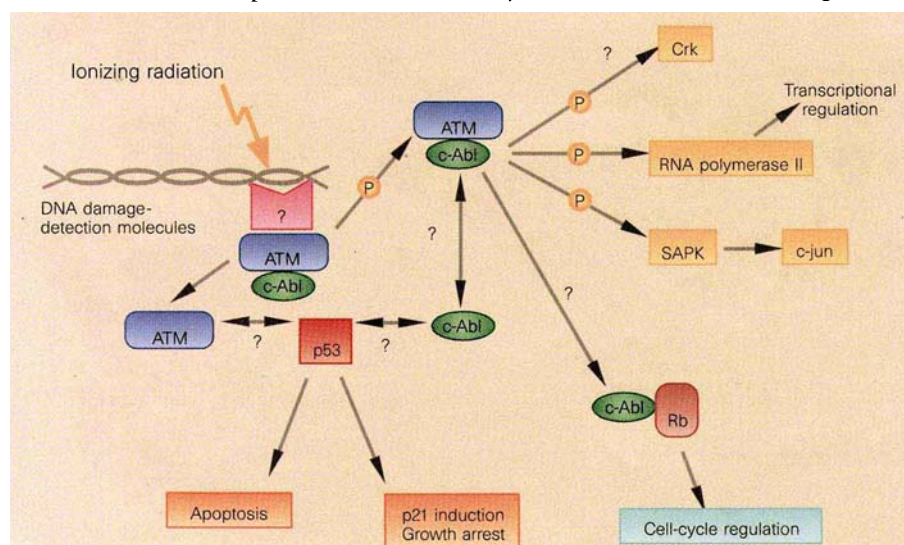


Figure 1 Pathways affected by the ATM (Ataxia telangiectasia mutated) protein after ionizing radiation. Baskaran *et al.*¹ and Shafman *et al.*² have found that ATM is constitutively bound to c-Abl and, following radiation-induced DNA damage, ATM phosphorylates c-Abl. Downstream targets of active c-Abl include RNA polymerase II, SAPK and Crk. ATM may also associate with p53 and the retinoblastoma protein (Rb), influencing cell-cycle regulation and apoptosis. 'p' indicates phosphorylation events.

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One step to Earth

Michael J. Walter

In the Earth sciences, Occam's razor is often dulled by the rock-hard reality of the complexity of geological processes. Models for Earth accretion are no exception. Did the Earth form homogeneously, with a continuous supply of material of essentially unchanging composition, and a single phase of differentiation into metallic core and silicate mantle? Or was accretion a complex, heterogeneous process with many stages? Until a few years ago the simpler model had been buried in favour of complex models of heterogeneous accretion, but homogeneous accretion has begun to re-emerge as a viable alternative, because of new experimental data on the partitioning of siderophile (iron-loving) elements. Mantle abundances of many moderately siderophile elements, such as nickel and cobalt, can be accounted for by homogeneous accretion and equilibrium core formation in a deep magma ocean^{1,2}. Now Righter and Drake³ show that the same is true of a highly siderophile element, rhenium (Re).

Siderophile elements in the upper mantle hold important clues to Earth's early history. They are typically divided into two groups: the moderately siderophile elements, or MSE (P, Fe, W, Co, Ag, Ni, Sb, As, Ge and Mo); and the highly siderophile elements, or HSE (Os, Re, Ir, Ru, Pt, Rh, Au and Pd). With the exception of gold, HSE are refractory and so should not be fractionated from one another by cosmochemical processes. Indeed, HSE occur in the mantle in the same relative proportions^{4,5} (Fig. 1) as in carbonaceous chondrites — primitive Solar-System material.

To test whether the core formed by a simple equilibrium segregation of metal from silicates, we need to find metal/silicate partition coefficients ($D^{M/S}$) for siderophile elements over a wide range of physical conditions. $D^{M/S}$ for the HSE are difficult to determine experimentally because the HSE have very low solubilities in silicates (solids or melts), especially at the low partial pressures of oxygen necessary for core segregation in the Earth. One tack is to measure the solubilities of pure HSE metals in silicate melts at one atmosphere and over a range of oxygen pressures, and to calculate $D^{M/S}$ using known or estimated thermochemical data for the equivalent solubilities in iron-rich metal⁶. Using this method, and assuming that metal segregation occurs close to the surface (at low pressure) and that $D^{M/S}$ are essentially constant with temperature, the observed abundances of Re, Ir, Pd and Au in the mantle are orders of magnitude greater than expected (Fig. 1).

Furthermore, calculated $D^{M/S}$ and measured sulphide/melt partition coefficients for HSE show that metal or sulphide

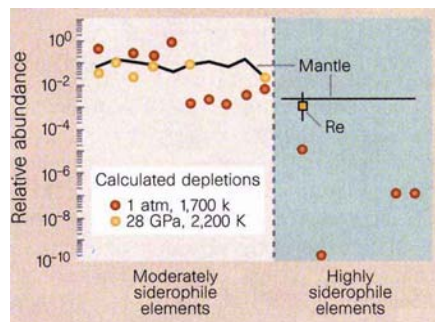


Figure 1 Depletion of iron-loving elements in the Earth's mantle. (Abundances are relative to those from primitive meteorite data.) Calculations of depletion at high pressures and temperatures match observed abundances well, including that of the highly siderophile element rhenium³. This supports a simple model of homogeneous Earth accretion, with the core and mantle separating in a deep magma ocean.

segregation to the core should have caused large fractionations among the HSE, resulting in non-chondritic relative proportions. So homogeneous accretion with equilibrium core segregation at low pressure appears not to explain observed abundances.

Heterogeneous accretion models, which successfully account for the abundances and relative proportions of HSE in the mantle, have reigned for well over a decade. They postulate that the first 93% or so of Earth's mass was primitive chondritic material accreted under reducing conditions. During this stage, siderophile elements were almost entirely scavenged into the iron-rich metal that segregated to form the proto-core. Then about 7% more of Earth's mass was added as more oxidized material, giving the present upper mantle its high oxidation state and contributing most of its volatile elements. This stage also re-enriched the mantle in HSE and MSE, but small amounts of metal and sulphide segregation to the core subsequently stripped the silicate mantle of HSE, while leaving MSE little changed. A final 'late veneer', less than 1% by mass, of oxidized but volatile-element-depleted material was added, bringing the HSE up to observed mantle abundances at chondritic relative proportions⁷. But no models explain adequately the origin of these proposed components of the solar nebula (from which the planets formed).

Conversely, in homogeneous accretion the material accreted to Earth had constant composition, on average, and metal segregation to form the core was an equilibrium process. In such models, the currently observed abundances of siderophile elements in the mantle reflect the conditions of core seg-

regation. In recent years, experimentalists have had much success in measuring the effects of pressure, temperature and composition on partition coefficients^{2,3,8-10} for many of the MSE. Righter *et al.*² parametrized available partitioning data for P, Fe, Ni, Co, W, Mo and S, and by extrapolation to high pressure, found that the abundances of these elements in the mantle can be accounted for by metal-silicate equilibrium in a magma ocean at around 28 gigapascals and 2,200 K, in general agreement with the values² previously inferred for Ni and Co. These results have made homogeneous accretion competitive with heterogeneous accretion, and imply that differentiation occurred deep in a magma ocean, rather than near the surface.

But can homogeneous accretion account for the HSE too? Righter and Drake³ show that rhenium abundances in the mantle can be accounted for by equilibrium metal segregation in a deep magma ocean and, within their uncertainties, for the same conditions as predicted for the MSE (Fig. 1). Although this is tremendous support for homogeneous accretion, the foundation is still shaky.

Chondritic relative proportions of HSE in the mantle place a very tight constraint on homogeneous accretion, because they mean that core segregation must have occurred under conditions where partition coefficients for all HSE were essentially the same, leaving them unfractionated in the mantle. Given the known variability⁶ in the chemistry of the HSE, this would be very surprising. It should be noted that there is considerable uncertainty in mantle abundances of many HSE, and it has been claimed that they may not have chondritic relative proportions¹¹. But recent isotopic data⁵ indicate strongly that the Archaean mantle had a chondritic Re/Os ratio. The critical next step is to determine $D^{M/S}$ for osmium, which is very challenging experimentally.

At least a new edge is beginning to form on the proverbial razor, and further work will determine whether it is needed to slice out a choice between the simple and the complex. □

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The mitochondrion that time forgot

Jeffrey D. Palmer

The mitochondrion, the eukaryotic cell's respiratory power plant for ATP generation, arose more than a billion years ago when a free-living eubacterium took up residence to the mutual benefit of eukaryote and bacterium. Since then, mitochondria have become nature's molecular playground. Just about every natural experiment known of tinkering with the ways in which genes and genomes evolve, and are organized and expressed, seems to have been tried in one or more mitochondrial lineages (see box on this page). The end result is that mitochondria from most major groups of eukaryotes vary substantially from one another, and from their eubacterial progenitors, in many aspects of their fundamental molecular biology¹⁻⁴.

A child who's missed this molecular playground has now been found. Lang *et al.* (page 493 of this issue⁵) report the complete sequence of the 69-kilobase mitochondrial DNA (mtDNA) of the heterotrophic flagellate *Reclinomonas americana*. This mtDNA is notable precisely because it is strikingly unelaborated — that is, truly primitive. As such, it forms a 'missing link' between its derived mitochondrial cousins and their relatively unchanging

eubacterial progenitors.

The astonishing feature of *Reclinomonas* mtDNA is its gene content. It contains 62 protein-coding genes of known function, far more than its nearest competitor (37 in the nonvascular plant, *Marchantia*), and dwarfing the puny genomes of ourselves (13), yeast (8) and the malarial parasite *Plasmodium* (only 3; see Table 1). These include all of the 44 protein-coding genes found variously in one or more previously characterized mtDNAs (but see table footnotes), plus 18 genes never before seen in mtDNA. Eleven of the 18 fall into categories already represented in other mtDNAs, but 7 represent four different biochemical functions that are wholly new to mtDNA (arrowed in Table 1). Two of these are especially noteworthy because they represent fundamental, classic eubacterial functions that have been entirely replaced in other mitochondrial lineages.

Gene transcription in *Reclinomonas* mitochondria is likely to be eubacterial-like, because its genome encodes all four components of a eubacterial-type RNA polymerase⁵. No RNA polymerase genes are found in any other mitochondrion, all of which are thought to use a nuclear-encoded,

single-subunit enzyme of the bacteriophage T7 type⁶. So it appears that, early in mitochondrial evolution, a phage-type polymerase was 'recruited', allowing loss of the RNA polymerase inherited from its proteobacterial ancestor.

The same may apply to another key mitochondrial function, protein sorting. The presence of a *secY* gene in *Reclinomonas* mtDNA⁵ implies that it uses a Sec-based pathway for sorting proteins within its mitochondrion, just as eubacteria and plastids do. Protein sorting in other mitochondrial systems, especially yeast, which lacks any detectable Sec homologues⁷, is thought to rely on separately acquired processes. Here again, *Reclinomonas* seems to have retained a fundamentally eubacterial process that has been supplanted by nuclear-encoded processes in other mitochondria.

Two other aspects of mitochondrial gene expression in *Reclinomonas* are much more eubacterial than in any other organism. First, Lang *et al.*⁵ predict that Shine-Dalgarno base-pairing occurs between the 3' end of *Reclinomonas* small-subunit ribosomal RNA and the 5' end of its messenger RNAs, whereas there is no evidence for this interaction in any other mitochondrion. Second, *Reclinomonas* mtDNA encodes an RNaseP RNA of highly eubacterial sequence and structure, whereas most mtDNAs don't even encode this RNA, and those that do specify highly divergent P RNAs. Beyond that, *Reclinomonas* mtDNA also retains essentially all

Deviancy in the mitochondrial genome¹⁻⁴

The first shockwave of mitochondrial deviancy came in 1979 with the discovery that our own mitochondria don't use the 'universal' genetic code¹⁰. Many more code deviations have since been found, especially within animals and fungi. Other unusual features of mitochondrial protein synthesis, some of which have evolved repeatedly, include expanded transfer RNA/codon-recognition patterns, use of tRNAs with highly modified structures, or which are imported from the cytoplasm or encoded by captured chloroplast genes, and use of highly evolved ribosomal RNAs, which are often greatly shrunken and occasionally highly fragmented. Many of these elaborations probably reflect

relaxed constraints operating on a translational system which specifies very few proteins.

The principal deviancy in gene transcription is a polymerase swap (see main text), while post-transcriptional processing has really gone wild. Diverse kinds of RNA editing have arisen on several occasions and, at the extreme, the transcripts of several kinetoplastid 'cryptogenes' acquire over half of their coding sequence through insertional editing. RNA splicing, of mobile group I and II self-splicing introns, is especially common in many fungal and plant genomes, reaching a zenith of complexity in the enormous genomes of plants where many group II introns are

fractured and must be trans-spliced from separate primary transcripts. Conversely, the compact genomes of vertebrates lack introns and rely on exquisitely precise processing to clip genome-length transcripts into monocistronic messenger RNAs whose termination codons are completed by 3' polyadenylation.

Mitochondrial genome size varies from only 6 kilobases to over 2,000 kilobases, even though all mtDNAs are vastly reduced in gene content compared with their eubacterial progenitors. Gene content is nonetheless still highly variable (see Table 1). Some mtDNAs retain major traces of eubacterial operons,

whereas others have scrambled their genes into solitary transcriptional units or newly formed 'operons'. Plant mtDNAs are notable for their dynamic, multipartite structures, as well as their frequent capture of foreign DNA from the chloroplast and nucleus.

Finally, mtDNA mutation rates are remarkably variable, being at least fifty times higher in mammals than in plants and defining the extremes known for DNA genomes. Furthermore, viewed through the lens of rRNA, most mtDNAs have independently moved into the evolutionary fast lane compared with eubacteria, causing profound problems in using rRNA for reconstructing eukaryotic phylogeny¹⁴. **J.D.P.**

of the primitive traits — use of the 'universal' genetic code, a nearly self-sufficient set of transfer RNAs of normal cloverleaf structure, a 5S rRNA, few introns (only one), an apparent lack of RNA editing, eubacterial-like operonic gene clustering — that have already been found in certain other mitochondrial systems, but which are highly modified in many others (see box).

What are the larger implications of the primitiveness of the *Reclinomonas* mitochondrial genetic system? Could it reflect a separate, perhaps recent, endosymbiotic origin? Probably not because, as Lang *et al.*⁵ point out, *Reclinomonas* shares with other mtDNAs two mtDNA-specific gene clusters that are unknown in eubacteria. Furthermore, that *Reclinomonas* mtDNA is itself highly reduced from a eubacterial progenitor with 500 or more genes, yet contains all of the protein-coding genes found variously in other, more reduced mtDNAs, fits best with a monophyletic origin of all mitochondria.

Or could *Reclinomonas* in general be unusually primitive, possibly representing a very early branch of eukaryotic evolution⁸?

Perhaps, but remember that the tree of extant life is 'right-justified' — that is, all extant organisms have had equal time to accumulate both primitive and advanced traits. So I expect further study to show that many non-mitochondrial traits of *Reclinomonas* are not primitive, while resolution of its phylogenetic position requires much more data, especially from nuclear genes.

Reclinomonas should now become an object of intense study — a model mitochondrion if not organism. Moreover, the wider search continues. *Reclinomonas* mtDNA was sequenced as part of a far-sighted, Canadian project (the Organelle Genome Megasequencing Program; <http://megasun.bch.umontreal.ca/ogmp>), which has already sequenced 11 mtDNAs from a diversity of protists, with work on many more genomes underway. In all, this programme, which in my view provides by far the best value (per base pair) of any genome-sequencing project, has been fabulously successful in both revealing the spectacular radiation of mitochondrial genetic systems and providing a rich source of sequence information to help unravel eukaryotic phylogeny.

Of course, an even more primitive mtDNA might still be found. After all, although it is gene-rich for mtDNA, *Reclinomonas* mtDNA really only looks like a depauperate plastid genome (like those of land plants). Might the next shock in mtDNA exploration be the discovery of the mitochondrial equivalent of the red alga *Porphyra*'s plastid genome, which encodes some 210 proteins, including enzymes involved in all sorts of metabolic processes unrelated to bioenergetics⁹?

Finally, Lang and colleagues' discovery⁵ only heightens the highly idiosyncratic picture of mitochondrial gene distribution across taxa (see Table 1), and emphasizes the need for further study of the processes that underlie such radical changes in gene content. Only four genes (*cob*, *cox1*, and those encoding small and large subunit rRNA) are common to all mtDNAs that have been examined, whereas many genes are present in only one or a few unrelated taxa, implying 7–10 independent losses for each. Much work is needed to understand the extent to which these patterns reflect several independent transfers to the nucleus; single ancient transfers, with retention of functional duplicates in both mitochondrion and nucleus allowing many subsequent losses; many losses without transfer, either due to gene substitution, biochemical substitution or a truly dispensable function; or secondary acquisitions by lateral gene transfer. In any event, these baroque gene-distribution patterns reinforce the notion that evolution is often anything but simple, especially where the mitochondrion is concerned.

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Table 1 Variation in the gene content of mitochondrial DNA

	Hs	Ms	Sc	Am	Mp	At	Cr	Cc	Pf	Ra	Gene losses
Genetic processes											
rRNA (16S and 23S)	2	2	2	2	2	2	2	2	2	2	None
rRNA (5S)	0	0	0	0	1	1	0	0	0	1	9
transfer RNA	22	2	24	25	27	22	3	23	0	26	
RNA component of RNase P	0	0	1	0	0	0	0	0	0	1	8
ribosomal proteins	0	0	1	1	16	7	0	4	0	27	
▀ elongation factor (<i>tufA</i>)	0	0	0	0	0	0	0	0	0	1	7
▀ RNA polymerase (<i>rpoA,B,C,D</i>)	0	0	0	0	0	0	0	0	0	4	7
Respiration											
complex I (<i>nad</i>)	7	7	0	7	8	9	5	7	0	12	
complex II (<i>sdh</i>)	0	0	0	0	2	0	0	3	0	3	8
complex III (<i>cob</i>)	1	1	1	1	1	1	1	1	1	1	None
complex IV (<i>cox</i>)	3	3	3	3	3	3	1	3	2	3	
complex V (<i>atp</i>)	2	2	3	3	4	4	0	3	0	5	
Protein import/maturation											
cytochrome c biogenesis	0	0	0	0	3	3	0	0	0	4	
▀ cytochrome oxidase assembly	0	0	0	0	0	0	0	0	0	1	7
▀ protein import (<i>secY</i>)	0	0	0	0	0	0	0	0	0	1	7
Number of genes: protein coding	13	13	8	15	37	27	7	21	3	62	
Number of genes: total	37	17	35	42	67	53	12	46	5	92	
Genome size (in kb)	17	17	75	57	187	367	16	26	6	69	
Number of introns	0	2	8	28	32	23	1	1	0	1	

The numbers indicate the number of genes present in each gene category for each of ten selected mtDNAs^{1–5,9,12}. The diagram at the top depicts a current view of the phylogenetic relationships among the organisms concerned, which are: Hs, *Homo sapiens*; Ms, *Metridium senile* (cnidarian animal); Sc, *Saccharomyces cerevisiae* (ascomycete fungus); Am, *Allomyces macrogynus* (chytridiomycete fungus); Mp, *Marchantia polymorpha* (nonvascular plant); At, *Arabidopsis thaliana* (flowering plant); Cr, *Chlamydomonas reinhardtii* (green alga); Cc, *Chondrus crispus* (red alga); Pf, *Plasmodium falciparum* (apicomplexan parasite); Ra, *Reclinomonas americana*⁸. The number of gene losses is inferred from an ancestral-presence/all-loss model, assuming a monophyletic origin of mitochondria, and taking into account data from all sequenced mtDNAs, not just those presented here. Arrows indicate biochemical functions not previously associated with mtDNA, but for which genes occur in *Reclinomonas americana*. Genes of unknown function or thought to be acquired by lateral transfer are excluded — that is, intronic genes¹³; *dpo* (ref. 14; F. Lang, pers. comm.); *mutS* (ref. 15; D. Wolstenholme, pers. comm.); and retrotransposons¹⁶.

Short precursor shortens memory

Mark P. Mattson and Katsutoshi Furukawa

After spending several hours in a shopping centre, a 44-year-old woman returns to the car park but cannot remember where she parked her car — nerve cells in regions of her brain that subserve learning and memory are relentlessly withering, and then dying. This is because she has Alzheimer's disease (AD) which, in her case, is caused by the substitution of a single base in the gene on chromosome 21 that encodes the β -amyloid precursor protein (APP). As well as neuronal degradation, this results in the accumulation of fibrillar, knot-like deposits of a 40–42-amino-acid fragment of APP, called amyloid β -peptide ($A\beta$).

Amyloid β -peptide is liberated from APP by enzymatic cleavage. The mutations in APP that cause AD have been shown to increase the amount of amyloid β -peptide that is produced by brain cells and/or to increase the amount of the long ($A\beta$ 1–42) form, which leads to greater fibril formation and neurotoxicity¹. As studies of cultured brain cells have shown that amyloid β -peptide can damage and kill neurons¹, scientists have tried to study these effects by producing 'transgenic AD mice' which express mutant APP. These mice accumulate amyloid β -peptide in their brains and show some behavioural anomalies, but they lack neuronal degeneration^{2,3}. Now, however, on page 500 of this issue, Nalbantoglu *et al.*⁴ report that they have used a different approach to create a potential ani-

mal model of AD, in which mice show — in an age-dependent manner — deposition of amyloid β -peptide, neuronal degeneration and deficits in short-term memory.

APP is a transmembrane protein with a large extracellular amino terminus and a short cytoplasmic carboxy terminus. The amyloid β -peptide sequence lies partially outside the cell and partially within the membrane. Full-length APP can be enzymatically cleaved at several sites. Cleavage in the middle of amyloid β -peptide releases a secreted form of APP (sAPP α), which modulates neuronal excitability and acts as a survival-promoting factor for neurons⁵. Cleavage at the extracellular amino terminus of amyloid β -peptide leaves a 100-amino-acid, transmembrane fragment (C100) which is further cleaved at the carboxy terminus, yielding intact amyloid β -peptide (Fig. 1).

Previous studies have shown that expression of C100 causes cultured neural cells to degenerate⁶. Nalbantoglu *et al.*⁴ used this knowledge to generate transgenic mice that express the carboxy-terminal 104 amino acids of APP. As these so-called C104 mice age, their short-term spatial memory (which is tested using a standard 'water maze' method) becomes impaired. The water-maze memory test requires an animal to use distal cues to swim to a platform that is hidden in a large pool, and demands proper function of neurons in the hippocampus — a region of the

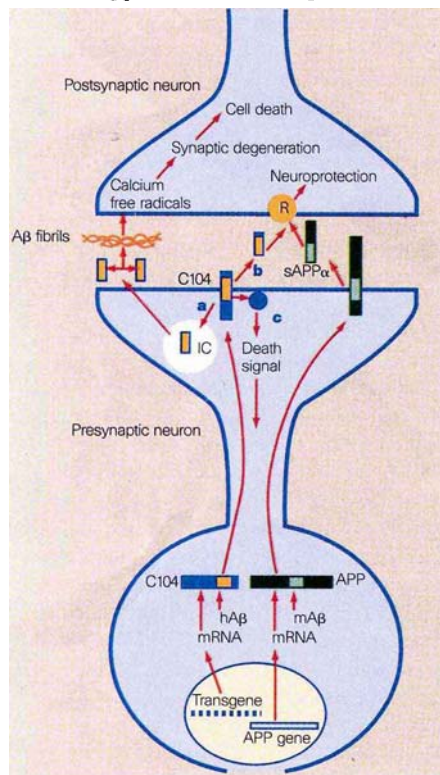


Figure 1 Nalbantoglu *et al.*⁴ have developed a new mouse model for Alzheimer's disease (AD). Neurons in the brains of C104 mice express both the normal, full-length mouse β -amyloid precursor protein (APP), which contains mouse amyloid β -peptide (mA β), and the 104-amino-acid carboxy-terminal fragment (C104) of human APP, which contains human amyloid β -peptide (hA β). The C104 fragment may promote synaptic degeneration and neuronal death, with resulting memory impairment, in one (or more) of the following ways. a, C104 is endocytosed and enzymatically cleaved in an intracellular compartment (IC), leading to the production of hA β which then forms fibrils. The amyloid β -peptide fibrils induce the production of free radicals and accumulation of calcium in neurons, both of which cause neuronal degeneration. b, C104 is cleaved in the middle of amyloid β -peptide (by α -secretase), releasing a small, amino-terminal fragment from the cell surface. This fragment interferes with the ability of the normal mouse secreted form of APP (sAPP α) to activate its receptor (R), which normally prevents neuronal degeneration. c, C104 interacts with a cytoplasmic protein, resulting in the activation of a signal-transduction cascade that induces cell death.



100 YEARS AGO

In your issue of May 13 (p. 46), a writer mentions *some Australasian boomerangs as thrown not to return* (if I have rightly understood him). I had not heard of these before, but in British India at least one race, the Kolis of Northern Gujarat, have the like. These are invariably of "fish" section, varying in weight, curve, and material; but the commonest and most efficient sort is of "Babul" wood (*Acacia arabica*), with the natural curve of the heart of the wood, something like that of an old-fashioned genuine Turkish sabre, rather a "knee" than any regular geometric curve. They are used with great effect on ground-game; much less, of course, on birds. In one case an old and feeble man, threatened by a swordsman, cut the assailant's shins across with his boomerang at about ten paces, and brought him down. Before the astonished thief could rise, the now unarmed old man had disarmed and literally taken him captive. ... Many Indian races throw straight sticks at game habitually, and even in a city riot the thrown sticks are often more dangerous than those in hand (bar iron-bound clubs). But their practice is not scientific, like boomerang-throwing. From *Nature* 27 May 1897.

50 YEARS AGO

The debate on the book publishing trade in Great Britain which took place in the House of Commons on the adjournment on May 9 reflected general disquiet on the subject. ... The shortage of paper supplies existed before the fuel crisis, and the unsatisfactory position in regard to scientific and technical books was discussed in *Nature* of December 28. Sir Stanley Unwin in a letter to *The Times* stressed the way in which even Government departments as well as the schools and colleges have now become handicapped by the scarcity of books, and referred to the inability of publishers to meet the insistent demand abroad for British books. ... All reserve stocks of paper for books, particularly school-books, have been used up, and with their present paper quota publishers cannot possibly begin to make up the present deficiency of books for schools or colleges, or of recognized classics, and at the same time continue publishing as a live industry. From *Nature* 31 May 1947.

brain that is ravaged in AD patients. Moreover, the authors found that the C104 mice show neuronal degeneration in the same region of hippocampus that is affected in AD.

Learning and memory involve communication between neurons at specialized structures called synapses. Here, a chemical messenger (neurotransmitter) is released from one neuron in a neural circuit, and it binds to cell-surface receptors on the partner neuron. When activity in hippocampal neurons is intense — such as when mice are being challenged with a memory task — long-lasting molecular changes occur at the synapse, so if that synapse is stimulated again its response will be stronger. By stimulating one set of hippocampal neurons and recording electrical activity from their postsynaptic partners, Nalbantoglu *et al.* showed that the normal strengthening of synapses does not occur in aged C104 mice.

The correlations between deposition of amyloid β -peptide, neuronal degeneration, synaptic dysfunction and impaired short-term memory in the C104 mice make them an important addition to the list of APP mutant mice. Last year, Neve and co-workers⁷ generated transgenic mice expressing C100, and examined their brains at both the light- and electron-microscope levels. They found age-related neurodegeneration and synapse loss in the hippocampus of the C100 mice and, although amyloid β -peptide deposition was not examined, these mice showed behavioural deficits. So the new findings in the C104 mice are similar to those of Neve's group. Important steps will now be to establish the mechanistic underpinnings of the different abnormalities in mice that express C100 or C104, and their relevance to the pathogenesis of AD in humans.

New findings often raise more questions than they answer, and the extent of amyloid β -peptide deposition in the C104 mice⁴ seems to be considerably less than in mice expressing mutant APP (refs 3, 4). Yet the C104 (and C100) mice show a loss of neurons in their brains, whereas APP mutant mice do not. So is amyloid β -peptide involved in the neurodegenerative process in C104 mice? To directly answer this question, the accumulation of amyloid β -peptide would need to be blocked in the transgenic mice. Nevertheless, there is circumstantial evidence implicating amyloid β -peptide in the neurodegeneration that is associated with AD: amyloid β -peptide damages and kills cultured neurons by inducing oxidative stress, impairing energy metabolism and disrupting calcium homeostasis⁸, and these findings are consistent with similar alterations that have been documented in the brains of AD patients.

Does expression of C104 in the transgenic mice promote neuronal degeneration by altering the normal trophic function of APP? Cleavage of C104 or C100 at the α -secretase

site would release a small fragment of amyloid β -peptide (Fig. 1), which may interfere with the trophic activity of full-length sAPP α (ref. 9). Alternatively, Neve and colleagues have proposed that C100 is linked to an intracellular signal-transduction pathway that induces neuronal death, and Suh¹⁰ suggests that C104 forms channels in membranes.

Transgenic mouse models of AD will provide crucial *in vivo* screening systems that may allow effective approaches towards the prevention and treatment of the human disorder to be identified. None of the current mouse models completely recapitulates the histopathological, biochemical and cognitive impairments that are seen in human AD patients. One glaring omission is the 'neurofibrillary tangle' — microscopic amalgamations of twisted filaments of a microtubule-associated protein called tau, within degenerating neurons. Tau is normally involved in regulating neuronal structure and function. Although certain brain insults in rodents (such as exposure to excitotoxins or physiological stress) can induce changes in tau that are similar to those seen in neurofibrillary tangles¹¹, the stable twisted tau filaments do not occur.

An extreme interpretation of the tau story is that none of the animal models so far established reflects the cellular and molecular mechanisms that are at work in AD. A more reasonable interpretation is that the underlying neurodegenerative mechanisms are relevant, but that there are differences in the properties of tau and/or its response to cell injury in rodents and humans. Indeed, analyses of cultured neurons¹² and transgenic mice¹³ expressing mutations in the *presenilin-1* gene (which is responsible for many inherited cases of AD) strengthen the case for the involvement of neurodegenerative cascades that include amyloid β -peptide and oxidative stress. The positive results obtained in clinical trials of antioxidants in AD patients¹⁴ indicate that the neurodegenerative mechanisms that have been proposed from studies of cultured cells and transgenic mice are indeed relevant — so car parks of the future may well contain fewer misplaced cars. □

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Daedalus

Dry water

Last week, Daedalus invented a surfactant that stabilized small bubbles. The hydrophilic ends of its molecules were bulkier than their hydrophobic ends; packed into a monolayer on an aqueous surface, they curved that surface concave. He is now reversing the strategy. His new surfactants have molecules with a bulkier hydrophobic end. A monolayer of them on a liquid surface bends it convex, and thus stabilizes small droplets. By careful design of the surfactant molecules, droplets of any desired radius can be stabilized.

One use will be for smokescreens and stage fog. Sprayed through a nebulizer, a solution of the new surfactant will form a long-lasting fog whose droplets cannot evaporate. But Daedalus wonders what will happen when such a fog settles out. Even when packed together, the droplets could not merge into a bulk liquid; their curved monolayer surfaces would prevent them. The result should be a novel 'powdered water' of tiny separate droplets.

Powdered water will be strange stuff. Like a mass of frictionless ball bearings, it could never be heaped up, but would fill a vessel or spread over a surface like a liquid. The resulting thin film would be an ideal lubricant. Spill water powder on the floor, and you would slide helplessly over the frictionless droplets and crash to the ground. Should you knock yourself out, your rescuers could slide you smoothly out of the room — that is, if they could find anywhere to stand themselves. Skating and similar sports, and the technology of chutes and conveyors, will be transformed. Shallow, enclosed tracks loaded with water powder, a sort of combined road and canal, might replace other forms of transport.

Similarly, water powder would make an ideal machine lubricant. The smaller the droplets, the greater the pressure required to deform them, and the heavier the load they could carry. Drops a micrometre across should be about ideal. A bearing with a similar clearance would accept a single layer of such droplets. It would then run as smoothly as the finest ball race, yet with a welcome degree of 'float' from the resilient rolling droplets.

Even poured into liquid water, water powder would not disperse. It would persist as a water-in-water emulsion, a suspension of giant micelles. In high concentration, it would form a rich, thick, creamy paste which would still be almost pure water. With a little crafty flavouring, it would make a splendid low-calorie artificial cream.

David Jones

The first genome from the third domain of life

Rebecca A. Clayton, Owen White, Karen A. Ketchum and J. Craig Venter

The main domains into which life-forms can be divided are the Eubacteria, Archaea and Eukarya. Complete genomic sequences of examples of the first two were published in 1995 and 1996^{1,2}. Now we have an example of the third.

The *Yeast Genome Directory*, published as a separate supplement to this issue of *Nature*, celebrates the first complete sequence of a eukaryotic organism, the budding yeast *Saccharomyces cerevisiae*. The genetic map of *S. cerevisiae*, constructed by the yeast community over the past decades³, is compared in detail with the physical map revealed by the nucleotide sequence. A catalogue of the predicted coding regions is included, along with a graphical view of their arrangement on each chromosome. The *S. cerevisiae* genome displays significant redundancy, with 53 duplicated gene clusters between the 16 chromosomes. These duplicated regions represent more than 30% of the entire genome⁴. Finally, the nucleotide sequences of chromosomes IV, V, VII, IX, XII, XIII, XIV, XV and XVI are presented to complement previous publications of chromosomes I, II, III, VI, VIII, X and XI⁵⁻¹¹.

The *Directory* is the first and only primary reference for the complete genome, which was first made available to the public on 24 April 1996 on the World Wide Web (http://speedy.mips.biochem.mpg.de/mips/yeast/yeast_genome.htmlx or <http://genome-www.stanford.edu/Saccharomyces>). This achievement is the result of a tremendous effort, in which over 600 scientists from 96 laboratories participated in world-wide programme, headed by André Goffeau, over a period of several years. The yeast genome project received much criticism in its early stages for its distributed character and its use of non-automated technology, but the fact that it now takes its place as the first completed eukaryotic genome effectively neutralizes these arguments. The yeast genome will be used as the reference genome for all eukaryotes, including humans.

In their introduction to the *Directory*, Mewes *et al.*⁴ describe the *S. cerevisiae* genome as an "indispensable resource for the detailed analysis of cellular gene function and genome architecture". This is certainly true, even though the *Directory* itself provides limited scientific analysis. The public availability of the complete genome sequence has already resulted in a number of yeast genome analyses published before the print appearance of the data.

One approach to whole-genome analysis involves compiling complete inventories of paralogous gene families, characterizing them and placing them in biological perspective. Paralogous genes are coding sequences within a genome that have arisen from duplication events. This type of study in *S. cerevisiae* is already bearing fruit. An inventory of protein kinase genes identified 113 sequences, 2% of the total genome, that could be classified into several families¹². Many identified yeast kinases that participate in signal-transduction cascades have homologues in other eukaryotes (homologues are proteins in different organisms that are derived from a common ancestor and have similar functions).

Analyses of yeast ATP-binding cassette (ABC) transporters (29 sequences)¹³ and multidrug-resistant transport proteins from the major facilitator superfamily (MFS-MDR, 28 sequences)¹⁴ have also been conducted. Several yeast ABC transporters have human homologues that are associated with disease, including YCF1, the yeast homologue to the human CFTR protein that is involved in cystic fibrosis, and PXA1, the yeast homologue to human ALD involved in adrenoleukodystrophy. By contrast, proteins in the MFS-MDR transporter family have well-characterized homologues in Eubacteria. For example, the yeast Car1, ybr008c, ybr043c and ybr180w proteins are similar to the product of the *bcr* gene in *Escherichia coli*, which confers resistance to bicyclomycin; and the yeast Atr1 and ybr293w proteins are homologues to the *Bacillus subtilis* protein for

methylenomycin resistance, encoded by the *mmr* gene.

Other studies have focused on the physical structure of the chromosomes and their biological relevance. In addition to the map analysis, presented by Cherry *et al.*³ in the *Directory*, it has been possible to describe and quantify periodic G+C-content variations along some chromosomes^{15,16}. These variations correlate with variations in gene density and recombination frequency. G+C-rich domains exhibit high gene density and, in chromosome III, they also correspond to areas of high recombination. Recombination frequency also varies between chromosomes. The smallest chromosomes (I, III, VI and IX) exhibit frequencies greater than the average calculated for the genome as a whole. Observations are also being made on the roles of transposons and the structures of telomeric regions in the yeast genome¹⁵, and the origin and significance of intragenomic gene duplication is undergoing redoubled examination now that a complete picture of the genome is available. Velculescu *et al.*¹⁷ have published a characterization of the yeast 'transcriptome' (the set of expressed yeast genes) using serial analysis of gene expression (SAGE), and have mapped expression levels across the chromosomes, demonstrating that gene expression is evenly balanced across the chromosomes, with no defined 'hot transcription spots'.

Faced with this wealth of new information about a time-honoured genetic 'model organism', Dujon¹⁶ has asserted that the most remarkable finding of the yeast genome project is the discovery of 2,000 yeast genes of unknown function, and with no homologues of known function. These 'orphan' genes are a vast virgin territory to geneticists and protein chemists. The desire to understand these orphans is also a powerful stimulus to the development and application of new technologies to study gene function. New approaches already being used include gene disruption directed by the polymerase chain reaction¹⁸, hybridization of probes on chips¹⁹, and SAGE¹⁷.

Mewes *et al.*⁴ point to the need for intensive analytical work on yeast genes of unknown function, the possibilities for interpreting the significance of gene location within the yeast genome, and the difficulties that repository databases face in coordinating functional information with sequences. We agree that much exciting intragenomic work remains to be done on the yeast genome. But the *S. cerevisiae* genome also has an important contribution to make to comparative genomics. The publication of the *Directory* represents a milestone in comparative biology — the first completely sequenced eukaryotic organism puts within our grasp whole-genome comparisons spanning the three

*The *Yeast Genome Directory* will be sent automatically to full-rate subscribers. Personal subscribers who have not completed one of the reservation forms that have appeared in recent issues of *Nature*, and who wish to receive a free copy of the *Directory*, should send an e-mail or fax to Sophie Ashworth (s.ashworth@nature.com; +44-171-843-4998) quoting their subscriber number.

domains of life, Eukarya, Eubacteria and Archaea.

Comparative genomics

The possibilities for intergenomic comparison are growing explosively. Seven complete genomes are currently available in public databases (*Haemophilus influenzae*, *Mycoplasma genitalium*, *Methanococcus jannaschii*, *Synechocystis* sp. PCC6803, *Mycoplasma pneumoniae*, *Saccharomyces cerevisiae* and *Escherichia coli*)^{1,2,20–22}. Based on information reported at the Small Genomes Conference, held in Hilton Head, South Carolina, in January 1997, as many as 30 projects for sequencing complete genomes are planned or underway. Some of these are listed on the World Wide Web site at our institution, <http://www.tigr.org/tdb/mdb/mdb.html>. It will be a challenging task indeed to present this amount of information to the scientific community and to convey the importance of comparative genomics to the general public. Simply cataloguing such an enormous amount of data will not be sufficient, and pairwise comparisons (for instance, searches for protein sequence similarities which identify the 'best' database match) will not be adequate methods for gene identification as the number of hypothetical proteins of unknown function increases exponentially in the databases.

Several topics emerge as fundamental to the comparative study of genomes. Is there a core set of genes for all organisms? Can any organism be considered a 'model system'? Does evolution proceed in the ancestor–descendant pattern that Darwin saw for multicellular animals and plants, or are there other patterns, such as those of lateral gene transfer, obscuring the ancient relationships between domains of life? There are two ways to approach these bioinformatics problems: global, genome-by-genome comparison (which is computationally intensive) and focused, system-by-system comparison (which is expert-analysis intensive).

Defining a core set of genes: global approaches

As an initial look at what might be done, we clustered protein sequences from the published complete genomes — specifically, *Saccharomyces cerevisiae* (a eukaryote), *Methanococcus jannaschii* (an archaean) and the eubacterial species *Mycoplasma genitalium*, *Mycoplasma pneumoniae*, *Haemophilus influenzae*, *Escherichia coli* and *Synechocystis* PCC6803 — on the basis of sequence similarity. From these sequence clusters, we extracted what we believe represent orthologue groups; that is, groups of genes from different organisms which have the same function. The clusters were initially defined by sequence similarity among the member

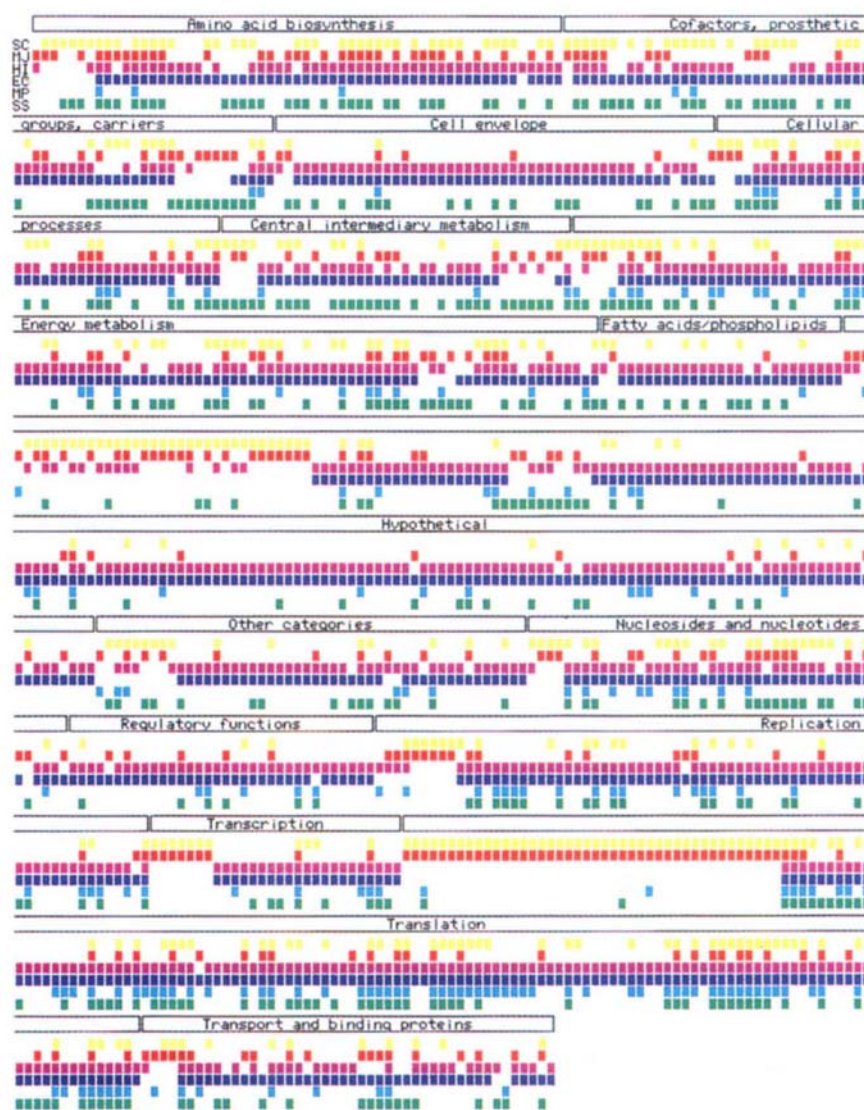


Figure 1 Orthologue groups among published genomes, specifically (top to bottom in each row) *Saccharomyces cerevisiae* (a eukaryote), *Methanococcus jannaschii* (an archaean) and the eubacteria *Haemophilus influenzae*, *Escherichia coli*, *Mycoplasma pneumoniae*, and *Synechocystis* PCC6803. Open reading frames were clustered here based on sequence similarity. These clusters were identified as orthologues (that is, proteins of similar function found in different species), and these orthologue sets were grouped by categories of cellular function. A surprisingly small number of genes have clear-cut orthologues in all six organisms. There are abundant orthologue groups including only the eukaryote, *S. cerevisiae*, and the archaean, *Methanococcus jannaschii*, in the categories of replication, transcription and translation. There are many other orthologue groups which include only members of the eubacteria, with no representative sequences from eukaryotes or archaeans.

sequences, and were then verified by comparing their putative functions, as defined by genome project annotations. (For most sequences, putative functions are based on similarity searches against sequence databases.) Figure 1 shows the computer-generated orthologue groups clustered by cellular role categories.

Of the computer-generated orthologue sets, only 53 groups contained representatives from each organism included in the analysis. These groups represent a core set of genes common to all of the organisms surveyed, but they certainly do not represent the full core set. Eighty orthologue groups

consisted of genes from yeast and *Methanococcus jannaschii* (Archaea and Eukarya, but no Eubacteria). As predicted by many archaeal researchers, there are abundant orthologue pairs between the eukaryote, *S. cerevisiae*, and the archaean, *M. jannaschii*, in the information-processing categories of replication, transcription and translation. These pairs have no obvious orthologues in the eubacterial genomes. Similarly, there are 504 orthologue groups with members from the eubacteria lacking archaean and eukaryote representatives.

As these orthologue groups are refined by further analysis, we expect to find that some

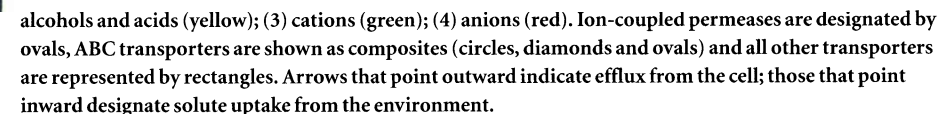


Figure 2 Membrane transport functions identified by analysis of coding regions in five complete genomes. Circular representations from the centre to the outer ring: *Mycoplasma genitalium*, *Methanococcus jannaschii*, *Synechocystis* PCC6803, *Haemophilus influenzae*, *Saccharomyces cerevisiae*. Colours represent the four role categories: (1) amino acids, peptides and amines (blue); (2) carbohydrates, organic

Table 1 Hypothetical proteins in completed genomes

Organism	% hypothetical proteins	Total hypothetical proteins
<i>Mycoplasma genitalium</i>	30	139
<i>Mycoplasma pneumoniae</i>	43	296
<i>Haemophilus influenzae</i>	38	656
<i>Methanococcus jannaschii</i>	55	962
<i>Synechocystis PCC6803</i>	55	1,754
<i>Escherichia coli</i>	60	2,583
<i>Saccharomyces cerevisiae</i>	56	3,480

The free-living organisms have between 55% and 60% hypothetical proteins (that is, proteins of unknown function). Even in the parasitic organisms (*Mycoplasma genitalium*, *M. pneumoniae* and *Haemophilus influenzae*), which presumably have reduced genomes, at least one gene in three is of unknown function. The predominance of uncharacterized proteins leads to a humbling realization: knowing the complete genome sequence for an organism is only a starting point for characterizing and understanding that organism.

exclusively eubacterial orthologue groups will prove to have highly divergent members from the Archaea and/or Eukaryota which our preliminary computer clustering overlooked. Some groups, currently identified as orthologues based on amino-acid sequence similarity, will be shown to have different functions. We also expect to see refinement in orthologue analysis techniques, as databases of biologically significant sites and patterns (such as PROSITE, BLOCKS and PRODOM) mature. Information on protein structure will also augment our ability to define orthologue groups accurately. Currently, only about 10% of proteins in each species have orthologues with known protein structure. As the protein structure databases grow, our definition of orthologues will evolve.

It is important to remember that there are many genes that do not appear in this diagram. Like the orphan *Saccharomyces cerevisiae* genes, these absentees appear to be unique to one genome. With such a small set of common genes, and so many unique genes, we are led to question the concept of *S. cerevisiae* or *E. coli* as model organisms — that is, physiological representatives of many other organisms. In a real sense, organisms that contain unique constituent parts cannot be models for one another. Having several whole genomes spread out for our examination underscores how different from one another these organisms are.

Whether or not they fall into orthologue groups, there is a huge number of new proteins of unknown function. Table 1 shows percentage and total numbers of hypothetical proteins for the completely sequenced organisms. The parasitic organisms *Mycoplasma genitalium*, *M. pneumoniae* and *Haemophilus influenzae* have smaller complements of hypothetical proteins, but the free-living organisms have between 55% and 60% 'hypotheticals'. The sheer magnitude of uncharacterized proteins underscores the fact that having the whole genome for an organism does not represent complete knowledge, but rather is a starting point

for characterizing and understanding that organism.

Transporter comparisons: system-by-system approach

Membrane transport phenomena have been highly conserved throughout evolution, and are well characterized in yeast and other organisms. Several gene families are known that encode homologous proteins in diverse species (ABC transporters, P-type ATPases and ion-coupled permeases, for example). We have chosen this system as an exemplar for what can be done with in-depth inter-genomic comparison.

We compared transport proteins from the annotated genomes of *Haemophilus influenzae*, *Mycoplasma genitalium*, *Methanococcus jannaschii*, *Synechocystis PCC6803* and *Saccharomyces cerevisiae* in four categories: (1) amino acids, peptides and amines; (2) carbohydrates, organic alcohols and acids; (3) cations; and (4) anions (Fig. 2). All organisms encode specific transporters for K^+ and PO_4^{2-} uptake, and at least one system for the extrusion of divalent cations. The autotrophic species, *M. jannaschii* and *Synechocystis PCC6803*, have a high proportion of proteins regulating ionic homeostasis. By contrast, solute transport in heterotrophs is dominated by systems for the import of metabolic substrates. The transport capacity encoded by the genome directly affects the biosynthetic potential of the organism and therefore dictates the environment each species can inhabit. Equally interesting is the functional diversity displayed by transporters of common structure. Genes encoding ion-coupled permeases are found in all four categories. Similar variety is observed in the bacterial and archaeal sequences for ABC transport proteins.

Beyond ribosomal RNA phylogenies

The technological advances that made feasible the sequencing of ribosomal RNA (rRNA) revolutionized prokaryotic systematics²³. Small-subunit rRNA sequences are currently the most widely used information-

al macromolecules in tracing evolution. Ribosomal RNA sequences obtained from uncultured environmental samples have made possible the discovery of groups of microorganisms^{24–26} new to science. Because so much rRNA sequence information has already been analysed, it is now possible for microbiologists to sequence the small-subunit rRNA of an unknown isolate and, by comparing it against the sequences in the Ribosomal Database Project²⁷, identify the most similar species.

Ribosomal RNA has been used for phylogenies at all taxonomic levels, from Ur-Kingdom²⁸ to subspecies. However, at the highest taxonomic levels, the burgeoning availability of DNA sequence for all domains of life offers opportunities to confirm or challenge the rRNA 'Tree of Life'. ATPase gene families²⁹, central intermediary metabolism phylogenies³⁰ and information-processing systems³¹ have already been explored with differing results. With the availability of complete genomes from all three putative 'Domains of Life', the possibility of fully exploring the evolutionary relationships at the base of the 'Tree of Life' finally exists.

The *Yeast Genome Directory* represents an enormous achievement for the many researchers involved. But its greater significance lies in the work that is yet to come. □

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Lichens, air pollution and lung cancer

The relationship between lung cancer and atmospheric pollution remains controversial¹⁻³ despite 50 years of discussion, partly because studies are frequently restricted to small, well-monitored areas. In contrast to instrumental monitoring, bioindication techniques allow the mapping of pollution effects over wide areas with a high sampling density. We have compared a biodiversity map of pollution-sensitive organisms, the lichens⁴, with mortality maps of a large part of northeastern Italy, the Veneto region (18,364 km², population ~4 million). Our results strongly support a relationship between air pollution and lung cancer.

The lichen study (data from 1991)⁵ was based on 2,425 measurements of epiphytic lichen biodiversity at 662 locations, calculated as the sum of frequencies of all species in a sampling grid of 10 units⁶. The mortality data at municipal level (1981-88) derive from the Italian National Institute of Statistics. Kernel indicators for the estimate of density functions^{7,8} were used for the analysis.

Biodiversity shows low, if any, correlation with several types of cancer (including larynx cancer, $r=0.016$), and with mortality by chronic bronchitis ($r=0.15$) because of the high mortality in mountain areas. There is no correlation with lung cancer in male migrants ($r=0.07$), or in resident women ($r=0.12$). Municipal data concerning women have a poor statistical quality

because lung cancer deaths in women are relatively few (13% of total lung cancer deaths), and there are pronounced differences in the smoking habits of women from rural and urban areas⁸.

However, biodiversity (Fig. 1a) and lung cancer in young (aged under 55 years) native male residents (Fig. 1b) are highly correlated ($r=0.82$, Fig. 2), even when corrected for spatial autocorrelation with bayesian analysis⁷. When all age-groups are included, the correlation becomes lower ($r=0.6$), owing to higher mortality of older men in mountain areas, many of whom emigrated between 1950 and 1970 to coal mines in Belgium.

We tested the hypothesis that lung cancer is correlated with lichen biodiversity as a result of air pollution, using pollution data recorded in nine municipalities since 1986. In these regions the correlation between biodiversity and lung cancer in young male residents was high ($r=0.95$, $P<0.001$). Furthermore, there was a high correlation with common anthropogenous pollutants, such as SO₂, NO₃, dust and SO₄²⁻ ($r=0.93$, 0.87 , 0.86 and 0.85 , respectively; $P<0.01$ in all cases); and no correlation with non-anthropogenous substances such as Cl⁻, Ca²⁺, Mg²⁺, HCO₃⁻, K⁺, Na⁺, or with all other types of cancer.

Lichens are notoriously sensitive to sulphur dioxide⁴, but the low SO₂ concentrations recorded in the survey area are unlikely to produce carcinogenic effects.

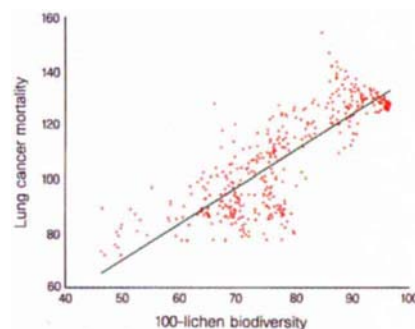


Figure 2 Scatter diagram relating lichen biodiversity (100 - sum of frequencies) and lung cancer mortality (observed/expected cases $\times$ 100; males aged under 55 years) in all municipalities of the Veneto region ($r=0.82$, $F=845.9$, $P<0.0001$).

However, the patterns of SO₂ concentration revealed by lichens do reflect the long-distance transport of different pollutants that may be emitted with SO₂, some of which may have carcinogenic effects.

The densely populated eastern and western parts of the Veneto plain are upwind and downwind of the main pollution sources, which may explain the low correlation between lung cancer in young males and population density ($r=0.23$). Pollution was higher between 1960 and 1980, but the main patterns of atmospheric transport have remained constant, indicating that time-lag factors are irrelevant.

The relative risk associated with pollu-

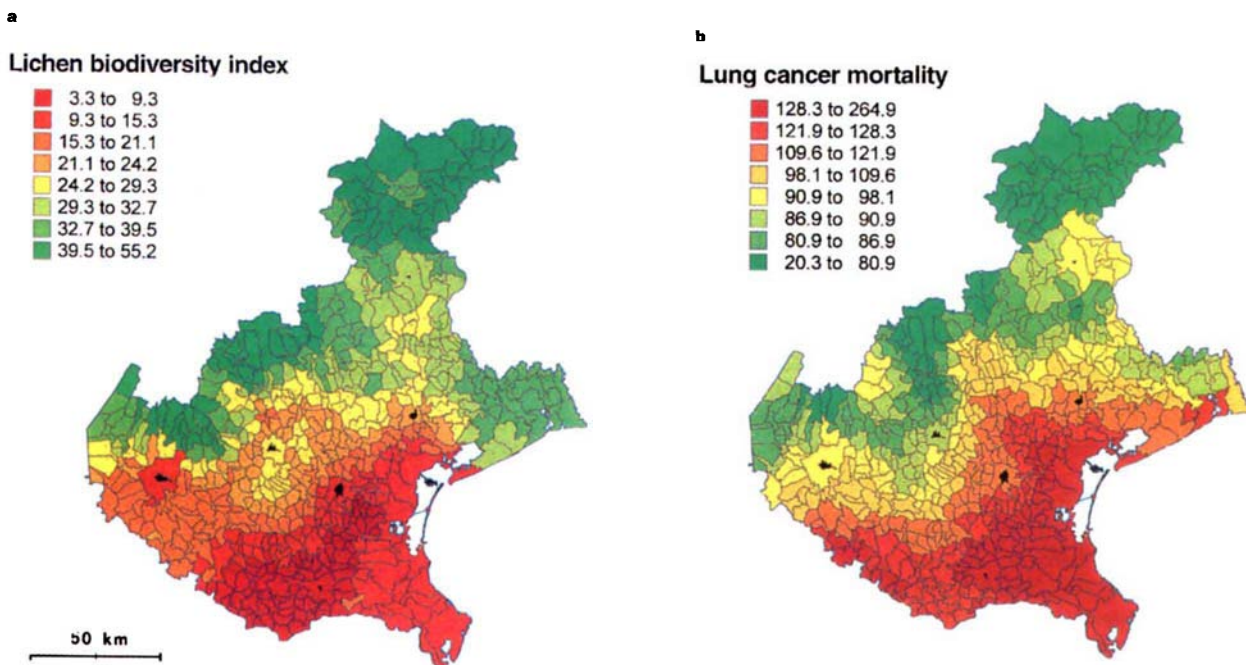


Figure 1 **a**, Lichen biodiversity, calculated as the sum of frequencies of all epiphytic species in a sampling grid of 10 units; and **b**, lung cancer mortality in young male residents (expressed as observed/expected cases $\times$ 100), in the region of Veneto. Scale intervals are based on percentiles of values distribution.

tion exposure is small, but the affected population is large, and thus the impact of pollution in terms of cancer mortality is important.

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Vervet monkeys as travelling salesmen

Vervet monkeys (*Cercopithecus aethiops*) forage on patchily distributed resources, visiting several sites in a single trip. When choosing a route, they face a famous optimization problem, the 'travelling salesman' problem¹. Here we pose a question about their route-choosing mechanism: how many future destinations along the route influence the choice of the next destination? We find that at least two further destinations affect the choice of the next destination, which is remarkable considering that the monkeys never visited more than six destinations in one excursion.

Our procedure was modelled on that of Menzel², who studied the travelling salesman problem in chimpanzees. He carried juvenile chimpanzees around while he hid fruit in 18 different locations, then released them and recorded the route taken to collect the fruits. They collected most or all of the fruits in sequences that bore no relation to the baiting sequence and that tended to minimize the distance travelled.

Our subjects were four adult female vervet monkeys selected from a 23-member troop living in a 9.15-m square enclosure. They were carried around to watch while grapes were hidden in six or eight small holes, randomly selected from 25 holes spaced at 1.53 m intervals in a 5 × 5 grid. Like Menzel's chimpanzees, our vervets ignored the baiting order and tended to minimize the distance travelled (Fig. 1a). However, they never visited more than six hiding places, confirming earlier reports that monkeys and apes differ dramatically in how many potential destinations they can keep in mind at once³.

A computer algorithm that always chose the closest site as the next destination did as well as our subjects, suggesting that such a mechanism would be adequate. However, performance on arrays chosen specifically to test for the influence of destinations beyond the nearest showed that the choice of the next destination was strongly influenced by at least two further destinations.

In the 'diamond' experiment, monkeys

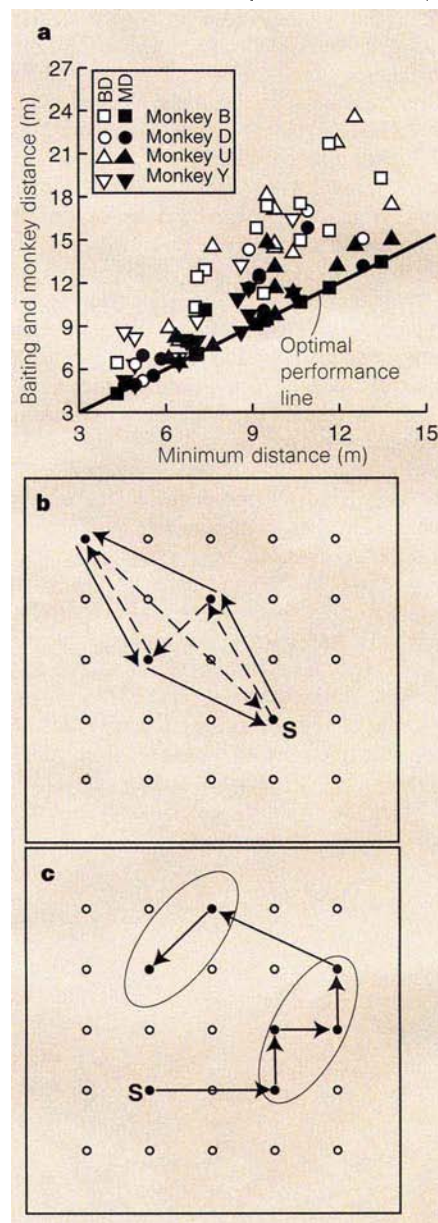


Figure 1 **a**, Distance travelled by the experimenter while randomly baiting food sites (BD) and the distance travelled by the monkey (MD) visiting these sites, plotted against the minimum distance by which all sites could be visited. **b**, A grape was put in the hole at the monkey's starting point (S) after it had reached the first visit site, requiring a return to the starting point later on in the visit sequence. The diamond route (solid arrows) is shorter than the zig-zag route (dashed arrows). **c**, Example of an unequal-sides configuration, with the two groups of visit sites enclosed in ellipses. Arrows indicate the monkey's route.

visited the four vertices of a diamond, one of which was the starting point (Fig. 1b). When the experiment did not require them to return to the start, an optimal route led first to both middle locations and then to the far end, but when the start was also a food site to which the monkey had to return, the optimal route led first to a middle location, then to the far point, and next to the other middle location on the way back to the start. Our subjects took the optimal (diamond) route in 20 of 26 experiments in which they returned to the start. By contrast, they visited both middle locations before the far location on 5 of 7 routes that did not include a return to the start.

A third experiment used four baited locations on one side of the enclosure, and two on the other (Fig. 1c), with the nearest location in each group being equidistant from the starting point. Our subjects chose to visit the side with the greater number of foraging sites first in all of 43 experiments. The decision to go first to this side must be dictated by at least two further destinations beyond the site nearest the monkey at the start. This result also indicates that factors beyond distance minimization might be important, for example, maximizing the harvest early in the trip. These considerations also require that future destinations affect the decision on which destination to visit next.

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Even odder carbons

In commenting on our recent observation that there are more organic compounds with an even number of carbon atoms than with an odd number (*Nature* **384**, 320; 1996), P. G. Stanley (*Nature* **385**, 782; 1997) suggests that "The pronounced excess of C-even compounds among those with large numbers of carbon atoms could be explained if a significant proportion of the compounds are dimers or other polymers". In fact, only a small proportion of the seven million compounds listed in Beilstein are dimers or higher polymers.

M. Kaser in the same issue points out that "Many of the naturally occurring organic compounds... are undoubtedly derivatives either of hexose sugars or of

aromatic rings." They are indeed, but this holds for compounds containing both odd and even numbers of carbon atoms. Only a tiny proportion of compounds are built exclusively from hexose sugars and/or aromatic rings, as would be required to explain the preference for multiples of six carbon atoms that is so striking in part c of our figure, and this applies to natural as well as synthetic compounds.

Biosynthetic routes such as the acetate pathway may account for the even-odd disparity in small subgroups of compounds but not in the vast Beilstein entirety. We have considered many factors that might contribute to the observed statistical trends, but cannot yet identify with confidence any single factor or particular combination of factors that would cover the totality of compounds of natural and synthetic origin.

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Rhodopsin guides fungal phototaxis

Rhodopsins act as the visual pigments of multicellular animals and also guide the swimming of green algae¹ towards or away from light (phototaxis). If these rhodopsin molecules have a common evolutionary origin, fungi, the sister group of animals², would also be expected to use a rhodopsin-based photoreceptor. Here we show that rhodopsin guides the zoospores of the fungus *Allomyces reticulatus*³ towards the light, suggesting that the origin of vision might have been the phototaxis of their unicellular ancestors.

The chytridiomycete *A. reticulatus* lives in the soil. Its phototactic zoospores (reproductive cells) swim with a single trailing flagellum or cilium (Fig. 1). There is a photoreceptor in the plasma membrane, which is screened from light passing through the cell by reddish lipid droplets localized near the base of the cilium to form a 'side-body complex'³ or eyespot. As in many algae, constructive interference increases the absorption of light from outside the cell⁴, so the 'eye' looks outwards and to the side. The pattern of light seen by

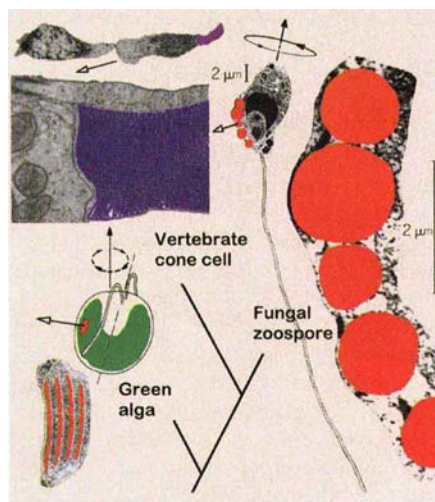


Figure 1 Rhodopsin photoreceptors of the green alga *Chlamydomonas reinhardtii*⁴, the fungal zoospore of *A. reticulatus*³, and an animal cone photoreceptor from *Felis domesticus*². Cells are shown at the same magnification and details of the eyes are magnified eight times. High refractive-index and absorbing lipid droplets are shown in red; receptor pigment in purple. Open arrows, direction of 'seeing'; closed arrows, direction of swimming.

the eye as the cell rotates provides the information for the cell to steer⁴. In some chytridiomycetes a specialized structure called a rumposome is crosslinked to a distinct area of plasma membrane^{5,6}, forming a more elaborate eye³.

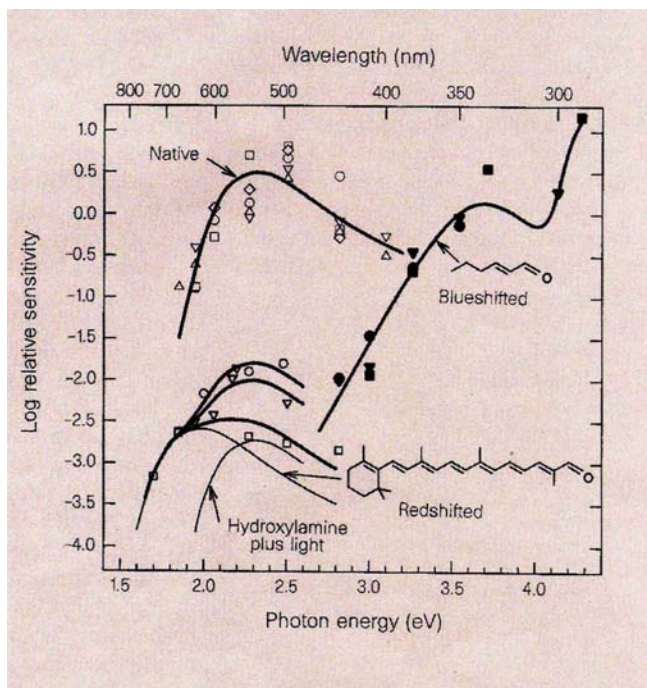
To test whether rhodopsin might be the visual pigment contained in the fungal photoreceptors, we measured the threshold

intensity for zoospores swimming towards different colours of light. Plotting the relative sensitivity (reciprocal of the detection threshold) against wavelength gives an action spectrum (Fig. 2). Differing chromophore absorption and protein environments^{7,8} can shift the peak absorption of rhodopsins over a wide range of wavelengths. Nevertheless, the native action spectrum for these zoospores fits the standard rhodopsin curve⁸ with a peak at 536 ± 4 nm, similar to a human green-sensitive cone.

N-retinylidene, the chromophore (light-sensitive constituent) of rhodopsins, forms by reaction of a retinaldehyde with a lysine residue of the opsin protein. Because retinal is synthesized from β -carotene we grew *A. reticulatus* for two generations in the presence of the carotenogenesis inhibitor norflurazon. These cells lost phototaxis sensitivity, but sensitivity was restored by the addition of all-*trans*-retinal, indicating that a retinal (of unknown isomerization) reacts to form the chromophore.

Light causes the rhodopsin chromophore to break down, releasing retinal. Hydroxylamine reacts with free aldehydes to form oximes, and can thus be used to remove any free retinal that might form a chromophore⁹. Without the bleaching effect of bright light, we found that hydroxylamine did not affect the sensitivity for phototaxis, but in bright light the presence of hydroxylamine caused the zoospores to be more than 1,000 times less sensitive

Figure 2 Threshold-phototaxis action spectra of *A. reticulatus* zoospores with its native chromophore and retinal analogues (structures are shown to the right of the curves). A video camera monitored the response of individual cells to unilateral light selected by blocked 10-nm-bandpass three-cavity interference filters¹. With untreated cells (the 'native' spectrum) five data sets (each indicated by a different symbol) were normalized to the same sensitivity and presented at their average sensitivity⁸. To incorporate retinal analogues, cells were treated with hydroxylamine (0.5 mM) and light (2.7 W, 500 nm, 32 nm bandwidth)⁹. Incorporation of *n*-hexenal¹ (0.6, 0.6, 2.5 μ M) resulted in the 'blueshifted' spectrum (the peak at 279 nm is due to absorption by opsin⁸). Incorporation of β -apo-12'-carotenal⁶ (10, 20, 20 μ M) resulted in the 'redshifted' spectra which are the combined spectra of the analogue and remaining native chromophores (shown as a thin line for the lowest spectrum). The data sets are normalized to the same sensitivity.



(Fig. 2). Retinal analogues (all-*trans*-retinal, *n*-hexenal, β -apo-12'-carotenal, and 2E,4E-octadienal) could all be incorporated to restore phototactic sensitivity of the bleached zoospores, showing that the loss of sensitivity was due to the loss of native chromophore.

Many retinal analogues¹⁰ have been bound *in vitro* to bovine opsin and bacterio-opsin, and *in vivo* to the opsin of the green alga, *Chlamydomonas reinhardtii*. These studies have revealed the chemistry of binding, the electric field and charge distribution within the binding site, and the shape of the site. Most retinal analogues shift the action spectrum away from that of the native pigment and also from the absorption spectrum of the unbound analogues, demonstrating their incorporation and function.

We compared retinal analogues bound in zoospores with the native chromophore that peaks at 536 nm. The analogue *n*-hexenal⁷ blueshifts to 339 $\pm$ 32 nm, β -apo-12'-carotenal⁸ redshifts to 626 $\pm$ 18 nm, and 2E,4E-octadienal blueshifts to 439 $\pm$ 11 nm (Fig. 2). The sensitivities and spectral shifts are comparable with their incorporation in *C. reinhardtii*, suggesting similarities in the binding sites and the interaction of the chromophores with their protein environments. The activity of these analogues and the corresponding shifts of their action spectra show that these zoospores use a rhodopsin to track light for phototaxis.

The use of rhodopsins in phototaxis by both green algae and fungal zoospores suggests that vision may have evolved from the phototaxis of a unicellular ancestor. Because they use the same type of photoreceptor, it is not surprising that some phototactic chytridiomycetes can gather in the same places as the green algae that they parasitize¹¹. Most important, a unicellular non-photosynthetic model system for a rhodopsin photoreceptor is now available.

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Uncertain turtle relationships

Turtles have generally been regarded as basal reptiles because they apparently retain the primitive anapsid skull pattern (no temporal fenestrae) unlike other extant reptiles which are diapsid (two temporal fenestrae on each side). However, the phylogenetic relationships between turtles and other amniotes is not well understood. Despite some disagreement over the details, recent phylogenetic analyses¹⁻³ seemed to be reaching a consensus that the turtles are the only surviving members of the Parareptilia, and thus only very distantly related to other extant reptiles. In contrast, by considering more morphological characters and more taxa than in previous studies, Rieppel and deBraga⁴ propose that turtles are crown-group diapsids. We have tested their claim that this parsimony analysis "robustly supports the diapsid affinities of turtles".

We reanalysed the data from ref. 4 with the PAUP⁵ software. Our unconstrained analysis replicated the results of ref. 4, yielding two most parsimonious trees with lengths of 770 steps in which the turtles are the sister group of *Placodus* and *Eosauropterygia* (position 1, Fig. 1). Remarkably, however, the shortest trees produced using topological constraints to force the turtles to lie within the Parareptilia required only three additional steps.

That such a small increase in tree length (<0.4%) is needed to include turtles within the Parareptilia is not consistent with robust diapsid affinities of turtles. Furthermore, pairwise statistical comparisons of constrained and unconstrained trees reinforce this lack of robustness. We compared the fit of the characters to the alternative trees using the null hypothesis that each character is equally likely to support either tree. Using the Templeton-Felsenstein test^{6,7}, as implemented in PHYLIP⁸, the most parsimonious trees in which turtles are parareptiles are not significantly less likely than those in which turtles are diapsids ($P > 0.05$). Application of the Wilcoxon signed ranks test⁶ supported this conclusion ($P > 0.74$, two tailed).

It is interesting that relationships between the other taxa are unaffected by the widely divergent positions of the turtles in the unconstrained and constrained trees, indicating that the turtles are a particularly problematic taxon to classify. Without doubt, the highly distinctive and divergent morphology of turtles is one major obstacle to inferring their phylogenetic relationships and evolutionary origins with much certainty. Another obstacle is the limited morphological data available for extinct putative relatives such as pareiasaurs and

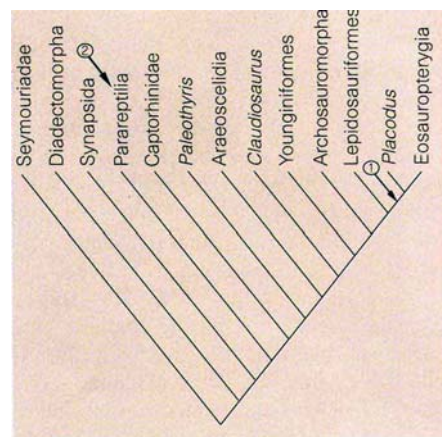


Figure 1 Amniote inter-relationships based on the data of ref. 4 showing the uncertain position of turtles. 1, Turtles as diapsids in unconstrained most parsimonious trees, requiring 770 steps⁴. 2, Turtles as parareptiles as posited in other studies¹⁻³, requiring only three extra steps.

placodonts, at least some of which must have extensive convergent similarities to turtles. Thus, barring the discovery of new and intermediate fossil forms, it seems unlikely that the available morphological data will prove sufficient to resolve the relationships of turtles convincingly.

The alternative hypotheses considered here (Fig. 1) differ in the relationships posited among the extant taxa, with either turtles more closely related to lepidosaurs (lizards) than to archosaurs (crocodilians) or archosaurs and lepidosaurs more closely related to each other than to turtles. They are thus amenable to testing using molecular and other neontological data. Molecular studies have yet to provide a well supported resolution of amniote relationships⁹⁻¹¹, but, simply by virtue of the potential wealth of sequence data, may yet provide our best chance of distinguishing between the alternative phylogenetic placements of turtles. Until such a time, phylogeneticists must be circumspect about the precise affinities of these enigmatic reptiles.

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Gifted discoverer of the neutron

The Neutron and the Bomb: A Biography of Sir James Chadwick

by Andrew Brown

Oxford University Press: 1997. Pp. 366.

£29.50, \$55

Brian Pippard

James Chadwick (1891–1974) was taciturn and little given to those merry quips, outbursts of rage or peccadilloes that can enliven a biography. The tale of so sterling a character, even when told as well as in this book, may be a little short on light moments, but any reader interested in the evolution of physics from an academic passion to a leading role on the world stage will find it a fascinating story and a worthy tribute to a great scientist.

Chadwick was nearly 48 when war broke out in 1939, at the height of his career and fully engaged in the construction of a cyclotron at the University of Liverpool, which was to be the most ambitious European venture in nuclear physics. Britain was then the leader in the first steps towards a nuclear bomb to counter the assumed parallel effort in Germany. The later decision by the United States to join the race, with far greater resources, provided much scope for jealousy on both sides, arousing real fear that only US citizens would be allowed to take part. It was Chadwick's mission to avert narrow-minded decisions that would have damaged both nations. It brought out in him a skill at diplomacy that few would have predicted, and in three years of unremitting toil tested, almost to the limit, his none-too-robust frame. He travelled to Los Alamos, New Mexico, in the middle of the war to work on the Manhattan Project.

General Leslie Groves, in overall charge of American nuclear bomb development, was a very different sort of man — large, blunt and autocratic, and utterly confident in his own judgement: qualities indispensable for success in the huge project. He quickly appreciated Chadwick's intellectual strength and personal integrity, as he had also recognized J. Robert Oppenheimer's high qualities, both men with characters foreign to his own. Chadwick and Groves found they could talk freely together, and their friendship helped to overcome the American desire to go it alone, which was by no means irrational in view of the genuine fear that security would be jeopardized by European involvement.

It was, of course, no accident that Chadwick was chosen to represent British interests. He was

already deeply engaged in the problems generated by the discovery of nuclear fission, and the first half of the book is concerned with his rise to a dominant position in nuclear physics from a very humble start. Little would be known of his early life if he had not talked freely in 1964 to Charles Weiner, who must have charmed him into uncharacteristic garrulity, even to speaking of the boyhood home on which he was usually silent. A scholarship took him to the University of Manchester, where he would have read mathematics but for the mistake of going to the physics lecturer for an interview and having his original intention subverted. Thus, in due course, he came under Ernest Rutherford's influence, which lasted until the great man's death in 1938.

Chadwick graduated and started research in Manchester. He won a scholarship from the Royal Commission for the Exhibition of 1851, only to learn that the commissioners insisted he hold it elsewhere. So in 1913 he went to Berlin for a year with Rutherford's old associate Hans Geiger; and in Germany he stayed, interned at Ruhleben as an enemy alien after war broke out, until November 1918. Life was hard, and his subsequent prolonged periods of ill-health may have started there. But the prisoners were resourceful, and organized educational courses and even small-scale researches with the help of friends among the German scientists. One of the group, a Woolwich cadet named Charles Ellis who had been caught on holiday, learnt about physics from Chadwick and on release went to Cambridge as a student, eventually joining Chadwick as a member of Rutherford's Cavendish Laboratory staff.

In 1918 Rutherford was still at Manchester and Chadwick returned to him there, but accompanied him in his move to Cambridge the following year. From then until 1935 he was Rutherford's right-hand man, calmly efficient in the administrative tasks that only irritated the professor, and at the same time taking a lead in the nuclear research that was the principal activity of the Cavendish as well as seeing to the needs of the ever-growing population of research students.

From the early 1920s, Rutherford had hoped to find evidence for a close association of the proton and electron which would act as a neutral component of nuclear structure, and in 1932 Chadwick made the actual discovery of the neutron which won him the

Nobel prize in 1935. By now the Cavendish was leading the world in nuclear physics, with an almost exclusively experimental approach that had been established by Rutherford but was very much to Chadwick's taste. Their experiments were economical in conception and their papers were notable for clear exposition and, as far as possible, avoidance of mathematical arguments — merits for which the author of this book must have been thankful. Chadwick's researches can be described without technical complication and still give a fair picture of what was achieved.

Despite Chadwick's preference for working alone with simple bench-top apparatus, Ernest Lawrence's invention of the cyclotron, and the consequent birth in America of a new style of nuclear physics with unprecedentedly massive and expensive equipment, persuaded him that Britain must follow suit or fall behind. Rutherford would have none of it, and his forthright rejection of Chadwick's proposals made a break almost inevitable. Chadwick went off to the chair at Liverpool and set about realizing his ambitions in a seriously run-down department. In the four years before the outbreak of war he had achieved much, and the cyclotron was just about working, but he had pressing calls on his time and never published any original work with it, leaving everything to his junior staff.

In fact, his career in academic research was over. By the end of the war, when he returned to Liverpool, he was exhausted and out of touch with the finicky details of university life under straitened conditions. Perhaps it was the hopes of rediscovering his lost Utopia that persuaded him to accept the mastership of Gonville and Caius College in 1948; but whatever the initial pleasures of a return to Cambridge, they were transient. Disagreements with the younger, and sometimes intolerant, fellows became more than he could bear, and at the end of 1958 he resigned. In those ten years he had not renewed contact with the Cavendish, and his departure from Cambridge was hardly noticed outside the college that had failed to appreciate his great gifts or to match his old-fashioned standards of civility.

Andrew Brown is a radiation oncologist and presumably not a stranger to nuclear physics. He has made a few slips in matters of detail which experts will correct without trouble and which are without consequence in the argument. He has made good use of what seem to be all the available archives except for a few still classified as secret. There is already a substantial collection of biographies of modern physicists, and this book is a fine addition to their number. □

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**Chadwick: great scientist
— and a diplomat.**

IMAGE
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REASONS

Ticking off

Telling Time: Clocks, Diaries, and English Diurnal Form, 1660–1785

by Stuart Sherman

University of Chicago Press: 1997. Pp. 323.
\$60, £47.95 (hbk); \$19.95, £15.95 (pbk)

Kristen Lippincott

For the benefit of the unwary who might find themselves seduced by the intriguing title, it should be pointed out that Stuart Sherman's book is essentially a work of literary criticism. The purpose of this warning is twofold: to save horologers, eager for another box-office hit like Dava Sobel's *Longitude*, from the spectre of false hope; and to alert those who might not be *au fait* with or sympathetic to current, post-Foucaultian theories of how literary texts ought to be read that they may find much of the rhetoric of this book impenetrable. For those willing to make the effort, however, there is one final warning — the central thesis of the book seems to be somewhat flawed.

Sherman argues that developments in clock and watch manufacture and decoration in England between 1660 and 1785 affected literary form during the period. At its simplest, it is a claim that has been made before and, in many ways, it is probably more-or-less true. Problems arise from the fact that the prompt for Sherman's particular point of view is a violent reaction to Frank Kermode's elegant formulation in *The Sense of Ending: Studies in the Theory of Fiction* (Oxford University Press, 1966). Kermode uses the analogy of the 'tick-tock' of a clock to illuminate the structure of a narrative where the anticipatory 'tick' of an unresolved situation is successfully brought to closure by the succeeding 'tock'.

To counter Kermode's poetry, Sherman calls three witnesses: the *Oxford English Dictionary*, John Aubrey's *Brief Lives* (1680) and the pendulum clock invented by Christiaan Huygens in 1656–57. From the first we learn that 'tick' is a word that has been known in England since 1440, whereas 'tock' was unrecorded until its appearance in 1848 in William Makepeace Thackeray's *Vanity Fair*. From the second, we note that a seventeenth-century watch is described as making a "tick-tick-tick" noise. And the third is cited as the "single, decisive" moment in the history of timekeeping, when the introduction of the pendulum as a constant driving force introduced synchronicity. "Huygensian time" was a new time because of "its capacity to track and report newly small durations — minutes and seconds — in regular, perceptible, continuous succession".

For Sherman, this "Huygensian time" is not "tick-tock time", which he claims did not exist until the nineteenth century. It is "tick-tick-tick time" which encapsulates "a temporality [his italics], a way of reckoning time

that includes but goes beyond counting, a way of conceiving it and experiencing it". Time for the English living during the second half of the seventeenth and first half of the eighteenth century was palpably different — even, constant, unending, unresolved. Pepys's *Diary*, the development of the daily newspaper and Daniel Defoe's travel writings all partake of this new "tick-tick-tick Huygensian time".

Sherman presents an interesting thesis, but it rests on one fundamentally erroneous assumption about clock mechanisms and their sounds. The "tick-tock" of a clock is caused by contact between the two pallets and the escape wheel. As the construction of these two pallets is never identical, the striking sound they make will always be different. And regardless of the driving force — whether by weight or spring — the "tick-tock" noise made by the alternating contact of the pallets has remained a constant feature of most public and domestic mechanical clocks and watches from the late thirteenth century to the present day. To my knowledge, there is only one type of escapement that could be claimed to make a "tick-tick" noise and that is the single-beat escapement first used around 1750 (although, in all honesty, the clocks with a detent escapement tend to make more of a "tock-pause, tock-pause" sound).

Furthermore, it seems somewhat ironic that many of the more sophisticated precision timekeepers of the seventeenth and eighteenth centuries — those whose supposed post-Huygensian "tick-tick" constancy should serve best to support Sherman's thesis — tend to reflect the complex structure of their movements in the sound they make. John Arnold's version of the single-beat escapement, for example, when closely analysed is, in fact, a delightful three-beat drum roll followed by a tiny click. And John Harrison's H-1 timekeeper veritably babbles with a "kerchunkety-kerchunkety-twang" — the twang being the expansion and contraction of the springs linking the interconnected balances.

One exaggerates to make a point, but it does seem somewhat precipitous for a scholar whose work is characterized by an extremely close reading of a given text to be so bold as to use analogies from other disciplines about which he is not sufficiently informed. His presentation of the history of chronometry and its relation to prose form simply does not stand up to scrutiny because his understanding of how clocks work and how timekeeping conventions changed during the seventeenth and eighteenth centuries is exceedingly patchy. The descriptions of the lunar-distance method of navigation are riddled with small inaccuracies and misunderstandings. And the account lacks an appreciation of some of the problems involved in time-reckoning between John Flamsteed's formulation of the equa-

tion of time and the introduction of standard time in Britain in 1850.

Literary critics may be convinced by Sherman's arguments, but from the perspective of a historian of science there is more than a little worry generated by claims such as "Pepys' ignorance is the crucial datum" because "...at Greenwich, Huygensian temporality will achieve hegemony, but not yet and not inevitably". One can only envy Pepys's ability to be so lucky in his ignorance. □

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Number crunching

Mathematics from the Birth of Numbers

by Jan Gullberg

Norton: 1997. Pp. 1093. \$50, £35

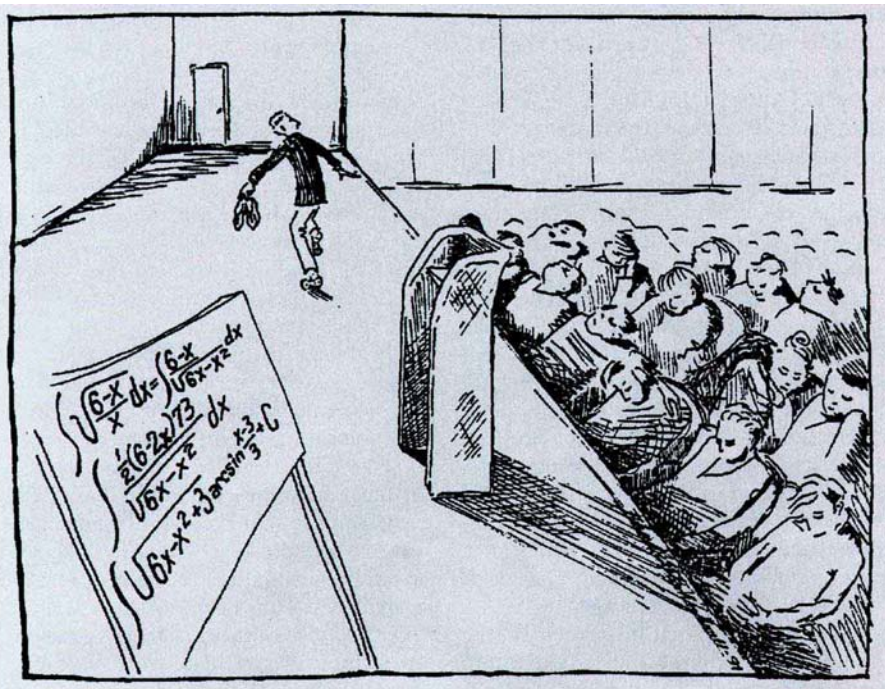
Peter Saunders

Most mathematicians find it very difficult to explain to other people what mathematics is and why we find it so absorbing. Even our students generally take some time to see the picture. They spend three or four years studying different parts of it in detail. While this is going on, we point out some of the connections and common features; others they become aware of by themselves. If all goes well, by the end of the course they have not only learned about the parts they have studied, they have also learned about mathematics as a whole.

Much of the skill in designing a good mathematics course consists of choosing and arranging the material in a way that will encourage this to happen, and much of the reason that many mathematicians are not especially enthusiastic about modular degrees is that they can seriously impede the process.

But what about people who do not have so much time to study the subject? How can they see how the different pieces are intertwined if they have not learned about them separately? How can they appreciate the way a theme recurs if they meet it only once? In short, how can they comprehend the unity of mathematics if they do not know enough about the subject? Yet it is the unity that is the real essence of mathematics, which makes it so much more than a collection of puzzles somehow combined with a set of clever methods for solving scientific problems.

Fortunately, one does not have to take a degree in mathematics to get a reasonably good idea of what the subject is about. One can manage with far less, but the material must be very carefully chosen to illustrate the key areas and to allow the connections to be made. That is easy to say, but difficult to put into practice. Authors have to think very hard about what to put in and how deeply it should be covered if they have only a few



Teaching mathematics is a daunting task.

hundred pages at their disposal and readers who are not going to work through large numbers of exercises. Skilful writing is called for; the reader has a lot of learning to do and needs clear explanations and illuminating examples.

This is a daunting task, even for a professional mathematician, but now an amateur, Jan Gullberg, has tried his hand. The idea does have its attractions: who better to tell the intelligent layperson about mathematics than someone who has had very much the same experience he wants his readers to enjoy? Unfortunately, he does not succeed.

Gullberg simply does not know enough mathematics to judge what constitutes a comprehensive survey. You can see this from his account of his own journey through mathematics. It was an impressive accomplishment for an amateur, but vast areas of the subject were left unexplored. Gullberg also has the disadvantage of never having taught the subject. Too often he gives the impression of simply having written everything he knows about a topic, rather than having selected the most significant and most revealing bits.

Important subjects are either rushed through in a page or two (such as the introduction of irrational and transcendental numbers) or ignored completely, while material that is less important and less interesting is covered in great detail. The most surprising omission is that there is no algebra beyond set theory and Boolean algebra. In the subject index, "ring" and "field" occur once each and "group" not at all, although there is a single historical reference to group theory.

There is little if any attempt to establish

links among the different topics that are included. Gullberg devotes a considerable amount of space to conic sections but, while he does inform the reader that they are literally sections of conics, he makes no attempt to establish that the two definitions are equivalent. He derives several different forms of the equations, but he neither carries out the classification of the conics nor even mentions that this can be done. Not only is this important, it is also quite easy once you know how to translate and rotate the coordinate axes, which Gullberg demonstrates immediately *after* he has finished with conic sections.

Gullberg seems to have set out to write at least two books at the same time: a modern *Mathematics for the Million* as well as *Calculus for Engineers*. The last 400 or so pages are therefore taken up with what amounts to an elementary methods course. The treatment is not very inspiring, and many techniques are presented with no attempt at justification, which is surely contrary to the spirit of the book. The choice of material is strikingly old-fashioned: there are many worked examples concerned with finding particular integrals and solutions in series, but nothing on qualitative analysis and stability.

The note on the dust jacket advertises the book as a "grand tour of the world of mathematics". I found it more like travelling with a guide who is himself a foreigner, who has learned some of the language but does not speak it fluently, and who misses out some of the most beautiful and important places because he does not know they are there. □

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A conquering man of genius

The Correspondence of Michael Faraday*

edited by F. A. J. L. James
Institution of Electrical Engineers, Michael Faraday House, Six Hills Way, Stevenage, Hertfordshire, SG1 2AY, UK

The History of the Faraday Society

by Leslie Sutton and Mansel Davies
Royal Society of Chemistry, Thomas Graham House, Science Park, Milton Road, Cambridge CB4 4UE, UK: 1996. Pp. 414. £20, \$55

John Meurig Thomas

Writing in a special issue of the *London Times* in August 1931 to celebrate the centenary of Michael Faraday's momentous discovery of electromagnetic induction, Lord Rutherford spoke for all scientists when he said: "The more we study the work of Faraday, with the perspective of time, the more we are impressed by his unrivalled genius as an experimenter and a natural philosopher. When we consider the magnitude and extent of his discoveries and their influence on the progress of science and of industry, there is no honour too great to pay to the memory of Michael Faraday — one of the greatest scientific discoverers of all time."

How very true, for in the chemical sciences he made seminal contributions to alloy steels, glasses, organic photochemistry, analytical chemistry, the liquefaction of gases, the laws of electrolysis, heterogeneous catalysis, fused salts and super-ionic conductors, plasma- and magneto-chemistry, colloidal metals and dielectrics. And in the physical sciences, apart from inventing the dynamo and the transformer, he established the identity of electricities from various sources (voltaic, static, animal, thermo) as well as investigating the discharge of electricity through gases, electrostatics, electrodeposition, and discovering the magneto-optical effect, which was the first proof that light had a magnetic component. On top of all this, he introduced the concept of a field. According to Einstein, Faraday (along with James Clerk Maxwell) was responsible for the greatest change in the axiomatic basis of physics since Newton.

But it is not just his extraordinary experimental skills and intellectual power that has made Faraday so fascinating to all succeeding generations; he also possessed intuition, insight and moral perfection. Aldous Huxley reflected on this "conquering man of genius" in 1925 and wrote: "If I could be born again and choose what I should be in my next existence, I should desire to be a man of science.... But even if I could be Shakespeare, I

*Vol. 1, 1811–31 (1991), pp. 673, £65; vol. 2, 1832–40 (1993), pp. 807, £65; vol. 3, 1841–48 (1996), pp. 835, £65.

think I should still choose to be Faraday. True, the posthumous glory of Shakespeare is greater than that of Faraday.... Posthumous fame brings nobody much satisfaction this side of the grave."

Do the three volumes of correspondence edited by James do justice to Faraday the man and Faraday the natural philosopher? And do they add that extra thrill for the reader who expects to be keeping company with genius? The short answer is yes. Some new insights into the extraordinary mental processes and inner thoughts of this remarkable former bookbinder emerge from these beautifully produced and meticulously researched volumes. The reader is also well served by the detailed introductions provided in each volume by James, who sets the scene in regard to the religious, social, public and private life of the great man. There is also a useful biographical register giving brief information on individuals mentioned three or more times.

For historians and philosophers of science, all this is perhaps as much as one should expect. But for the practising scientist — and every experimentalist can profit by burrowing into the correspondence of Faraday — what is lacking are inserts between letters giving descriptions of the key experiments (and other activities such as the arduous preparations for the Royal Institution Christmas lectures) that occupied Faraday's thoughts at that particular time.

Take, for example, Letter 522, addressed to Richard Phillips in November 1831 when Faraday begins: "For once in my life I am able to sit down and write you without feeling that my time is so little that my letter must necessarily be short....". Most readers would be unaware that in the ten preceding weeks Faraday had worked frenetically to sort out an explanation for electromagnetic induction, an intellectual achievement (expressed non-mathematically) that later drew excep-

tional praise from Maxwell and others.

Again, the period between January 1832 and December 1834 was among the busiest in Faraday's life. In this short time he published some 20 original papers, delivered 17 Friday evening discourses at the Royal Institution on an extraordinary range of topics, gave the six Christmas lectures (on chemistry) and 48 daytime lectures on a wide range of chemical and physical topics at the institution, and delivered 75 lectures to the Military Academy at Woolwich. He also found time for extensive correspondence (included in James's compilation) with many eminent people: Ampère, Gay-Lussac and Hachette in Paris; the editor of the *Literary Gazette* (expressing his annoyance about attribution of priority as between Nobili and himself for work on electricity and magnetism); Fazzini in Naples; Forbes in Edinburgh; Berzelius in Stockholm; Plateau in Brussels; and Mary Somerville, J. F. W. Herschel, Babbage, Barrow (secretary of the Admiralty) and Whewell in England. But in this period he made two landmark discoveries: the laws of electrolysis and the equivalence of electricity from different sources. If the editor had inserted information about this kind of scientific action of Faraday's between the appropriate letters, the result would have been compelling and *The Correspondence* greatly enhanced.

Some of the letters make especially interesting reading, not least those to Ada, Countess Lovelace (Byron's daughter), who seems to have admired Faraday to the point of infatuation. She proposed to assist him, and wanted to repeat all his key experiments under his guidance. Faraday's tact in coping with this problem, as with numerous others, is impeccable. Writing to Mary Somerville in 1834 he begins: "I cannot refuse myself the pleasure any longer of thanking you for your kindness in sending me a copy of your work". To the chemist Berzelius in 1832 he writes: "We have

been alarmed here by reports of your death but rejoiced again by finding it was false".

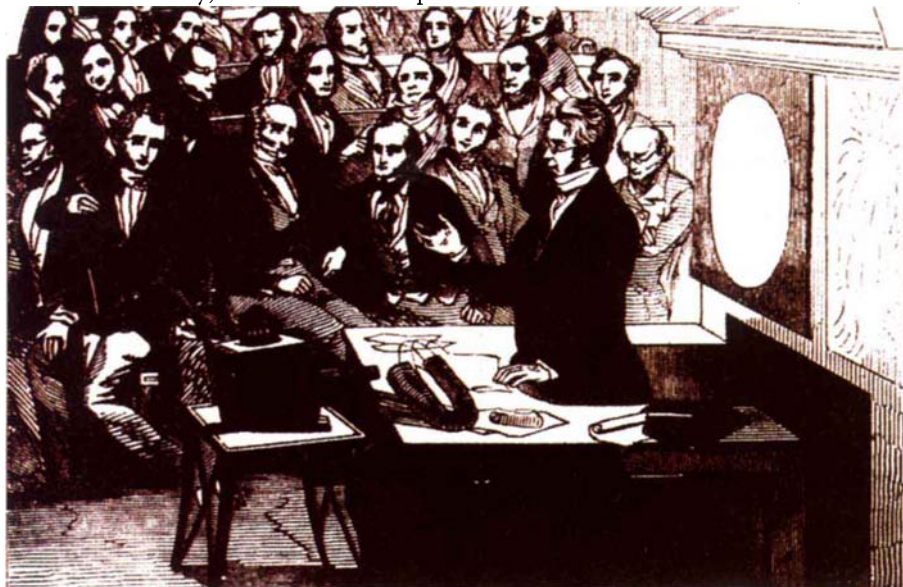
The letters of Faraday are remarkable not only for their vivacity and freshness but for their elevated tone and excellent composition — they are true specimens of the lost art of letter-writing. Bence Jones realized this as early as 1870 when he edited two volumes of them, and so did Pearce Williams, Fitzgerald and Stallybrass in their definitive two volumes (1971). Many of the best letters in the volumes under review have appeared previously; indeed, of the first 300 letters, the vast majority have already been published. There are, however, new letters, especially in the later volumes, not hitherto seen.

The *History of the Faraday Society* is an altogether different kind of book, full of factual information about the imaginative way in which the society came to set up what are still arguably the best kind of scientific conferences — the biannual Discussions. All registered delegates to a Discussion, on a timely topic selected by the society's council, are sent preprints of the 24 or so internationally representative papers two weeks before the meeting. At the event itself, the authors spend no more than five minutes summarizing the key points and then engaging in some 15 minutes of well-informed discussion. The meeting opens with an introductory lecture given by a world authority. All verbal discussion is recorded and is often supplemented by communications from active participants and anyone in possession of the preprints. Senior overseas contributors often make notable impressions, especially those who normally publish in languages such as Russian, Japanese or Chinese. These meetings have always attracted a galaxy of protagonists.

We read that at the Discussion held in Oxford in 1927 on "The theory of strong electrolytes", Debye was unavoidably absent, but active participants included Brønsted, Bjerrum, Fajans, Harned, Hevesy, Scatchard, Onsager, Christiansen, R. H. Fowler, Kraus and McInnes — all the major world figures. And at the Discussion in London in 1936 on "Electrolytes", Joel Hildebrand of the University of California, Berkeley, who gave the introductory lecture, was so impressed by the pellucid comments made by the 36-year-old J. D. Bernal on hydrogen bonding that "he asked the chairman whether Bernal could be obliged to leave the room immediately, to write out in full what he had just conveyed to the audience".

Leslie Sutton and Mansel Davies, both in their different ways pioneers of British physical chemistry, have written a delightfully evocative book redolent of the old-world charm and vitality that is still strongly associated with Faraday Discussions. □

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Energetic lecturer: Faraday giving one of his Friday evening discourses at the Royal Institution.

Measurement of the quantum states of squeezed light

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A state of a quantum-mechanical system is completely described by a density matrix or a phase-space distribution such as the Wigner function. The complete family of squeezed states of light (states that have less uncertainty in one observable than does the vacuum state) have been generated using an optical parametric amplifier, and their density matrices and Wigner functions have been reconstructed from measurements of the quantum statistics of their electric fields.

A central theme in many fields of quantum physics is the development and application of theoretical and experimental tools for obtaining information about the states of quantum fields of matter and radiation. Although the state of an individual particle or system is unobservable, it is possible to determine the state of an ensemble of identically prepared systems by performing a large number of measurements¹. Notable experimental success has recently been achieved in generating and determining states of various quantum-mechanical systems, employing newly developed methods of quantum state reconstruction (QSR)²⁻⁹. A single mode of light¹⁰⁻¹⁴, vibrational modes of a diatomic molecule¹⁵ and of an ion in a Paul trap¹⁶, and the motional state of freely propagating atoms¹⁷ have been characterized completely by determining their density matrix or, equivalently, their Wigner functions, a quantum-mechanical analogue of the classical phase-space distribution¹⁸.

A single spatial monochromatic mode of light represents a harmonic oscillator system for which non-classical states can be generated very efficiently using the interaction of laser light with nonlinear optical media. Squeezed states¹⁹, first generated about ten years ago^{20,21}, have a reduced uncertainty in a specific quadrature (for example the amplitude quadrature) compared to that of the vacuum state. They have typically been characterized by measuring the variances of the electric field with a homodyne detector. A complete investigation of their quantum features, in particular their photon statistics (which at present cannot be measured directly owing to technical limitations of available photon counters) has only become possible through the recent development of theoretical tools for QSR. First experimental investigations analysed coherent and squeezed vacuum states^{10,11,13,14}. Here we present a study of all types of squeezed states of light; squeezed vacuum, amplitude-squeezed states, phase-squeezed states and states squeezed in an arbitrary quadrature. For each of these states we construct 'portraits' in terms of both the Wigner functions (which are two-dimensional maps in appropriate phase-space coordinates) and the density matrices. These portraits contain all that one can know about the quantum-mechanical properties of the squeezed optical states.

Optical homodyne tomography

How is the quantum state of an optical wave determined? The measurements to be performed on the state are measurements of the electric field operator $E(\theta) \propto X_\theta = X \cos \theta + Y \sin \theta$ at all phase angles θ . Here $X = (a + a^\dagger)/\sqrt{2}$, $Y = (a - a^\dagger)/\sqrt{2}i$ are the non-commuting quadrature operators of the electric field, with a and $a^\dagger$ being the annihilation and creation operators. X and Y are analogous to position and momentum operators of a particle in a harmonic potential. To access experimentally the electric field, which oscillates with a frequency of $\omega/2\pi$ of hundreds of THz, a balanced homodyne detector²² is employed (see Fig. 1). In this

detector, the signal wave is spatially overlapped at a beamsplitter with a local-oscillator wave of the same frequency. The two fields emerging from the beamsplitter are proportional to the sum and the difference of the signal and local-oscillator fields. By detecting the difference of their fluxes, the natural oscillation of the signal state under investigation is converted to a low-frequency electrical signal I , which measures X_θ , where θ is the relative phase between signal and local oscillator. A large number of measurements of the observable X_θ yields the probability distribution $P_\theta(x_\theta)$ of its eigenvalues x_θ . This procedure is repeated for a set of different phase angles $\theta \in [0, \pi]$.

The relation between the measured distributions and the density operator ρ is $P_\theta(x_\theta) = \langle x | U^\dagger(\theta) \rho U(\theta) | x \rangle$, where $U(\theta) = \exp(-i\theta a^\dagger a)$ performs a rotation in phase space. As the optical state evolves freely with ω , U is equivalent to the time evolution operator with $\theta = \omega t + \text{constant}$, and the θ -dependence of P_θ is equivalent to the time dependence of the position probability density of the state (that is, of $|\psi(x, t)|^2$, if $\rho = |\psi\rangle\langle\psi|$ is a pure state). Thus, homodyne detection maps out the time evolution of a harmonic oscillator state. Our measurements (shown below) may be regarded as an implementation of the oldest example of quantum dynamics, the motion of a wavepacket in a harmonic potential studied by Schrödinger in 1926²³.

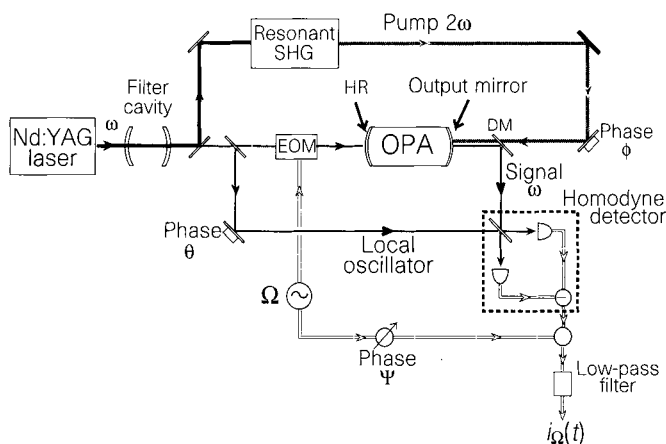


Figure 1 Experimental scheme for generating bright squeezed light and squeezed vacuum with an optical parametric oscillator (OPA). The electric field quadratures are measured in the homodyne detector while scanning the phase θ . A computer performs the statistical analysis of the photocurrent i_Ω and reconstructs the quantum states. EOM, electro-optic modulator; DM, dichroic mirror; SHG, second harmonic generator; HR, high reflector.

Of the various methods that have been proposed to reconstruct the quantum state numerically from the set of measured distributions P_θ , two are employed here. The first method makes use of the fact that the distributions $P_\theta(x_\theta)$ are the marginals of the Wigner function $W(x, y)$ in rotated coordinates;

$$P_\theta(x_\theta) = \int_{-\infty}^{\infty} W(x_\theta \cos \theta - y_\theta \sin \theta, x_\theta \sin \theta + y_\theta \cos \theta) dy_\theta \quad (1)$$

where $y_\theta = -x \sin \theta + y \cos \theta$. Therefore $W(x, y)$ can be obtained from the set P_θ by back-projection via the inverse Radon transform². The second method furnishes the elements of the density matrix in the Fock basis via integration of the distributions P_θ over a set of pattern functions^{3,4}. In contrast to the inverse Radon transform, this procedure does not involve any filtering of the experimental data and also allows an estimation of the propagation of statistical errors.

The experiment

The experimental set-up is shown in Fig. 1. Central to the experiment is a monolithic standing-wave lithium-niobate optical

parametric oscillator (OPA)^{13,24}, pumped by a frequency-doubled continuous-wave Nd:YAG laser (1,064 nm). The infrared laser wave is filtered by a high-finesse mode-cleaning cavity, which transmits 75% of the laser power. Its narrow linewidth of 170 kHz suppresses the high-frequency technical noise of the laser, yielding a shot-noise-limited local oscillator for light powers in the milliwatt range at frequencies ≥ 1 MHz (ref. 13). The pump wave 2ω (power ~ 20 – 30 mW) for the OPA is generated by resonant second harmonic generation.

In the past OPAs have been frequently used as sources of non-classical light^{10,13,25–28}. Operated below threshold, the OPA is a source of squeezed vacuum. We studied the field's spectral components around a frequency offset by $\Omega/2\pi = 1.5$ or 2.5 MHz from the optical frequency ω , to avoid low-frequency laser excess noise. To generate bright light (that is, with non-vanishing average electric field at the frequencies $\omega \pm \Omega$), we employ the OPA in a dual port configuration²⁸. A very weak wave split off the main laser beam is phase-modulated by an electro-optic modulator (EOM) at the frequency Ω (modulation index $\beta \ll 1$) and injected into the

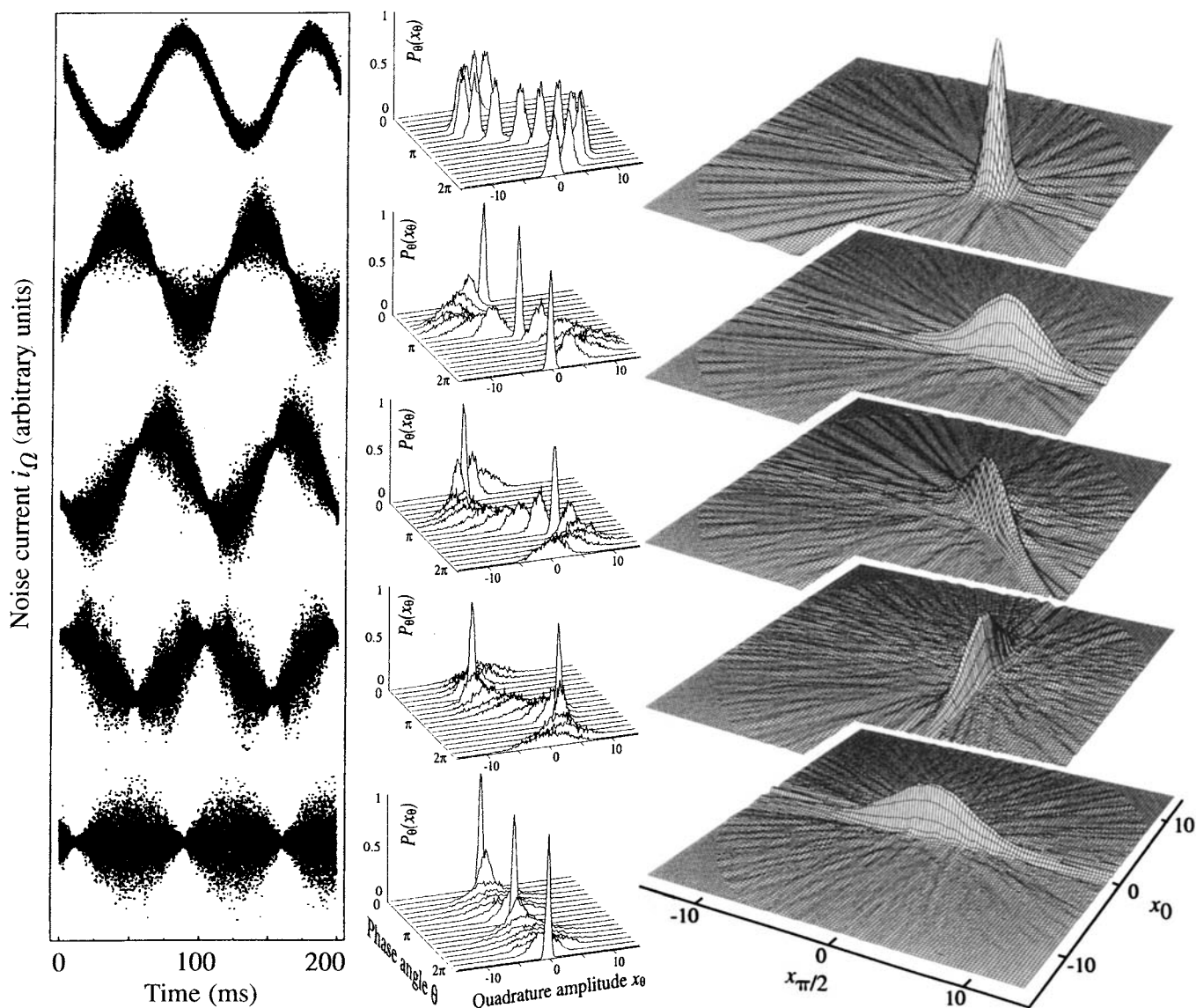


Figure 2 Noise traces in $i_Q(t)$ (left), quadrature distributions $P_\theta(x_\theta)$ (centre), and reconstructed Wigner functions (right) of generated quantum states. From the top: Coherent state, phase-squeezed state, state squeezed in the $\phi = 48^\circ$ -quadrature, amplitude-squeezed state, squeezed vacuum state. The noise traces as a function of time show the electric fields' oscillation in a 4π interval for the upper

four states, whereas for the squeezed vacuum (belonging to a different set of measurements) a 3π interval is shown. The quadrature distributions (centre) can be interpreted as the time evolution of wave packets (position probability densities) during one oscillation period. For the reconstruction of the quantum states a π interval suffices.

OPA through its high reflector (HR) port. The carrier frequency ω is kept on-resonance with the cavity and the two 'bright' sidebands $\omega \pm \Omega$ are well within the cavity bandwidth $\Gamma/2\pi = 17$ MHz (HWHM). In the semiclassical picture we may write the Fourier components at the frequency Ω' of the field's quadratures emitted from the output mirror as $X(\Omega') = E_0(\Omega') + \beta E_0(\delta(\Omega' - \Omega) - \delta(\Omega' + \Omega)) + X_n(\Omega')$, $Y(\Omega') = Y_n(\Omega')$, where δ is the Dirac delta-function, E_0 is the amplitude of the emitted wave and X_n , Y_n are the broad-band quantum fluctuations²⁹. Due to the very small ratio of HR transmission ($<0.1\%$) to output mirror transmission (2.1%), the transmitted sidebands and their quantum fluctuations are strongly attenuated. The quantum fluctuations of the signal wave inside the resonator originate essentially from the vacuum fluctuations entering through the output coupler. The injected seed-wave amplitude as well as the fluctuations are modified inside the resonator by the interaction with the 2ω pump wave: the quadrature fluctuations out-of-phase with the pump are deamplified (squeezed), the in-phase quadrature fluctuations are amplified. Similarly, the seed wave is deamplified if it is out of phase, and amplified if it is in phase, with the pump wave. As the relative phase ϕ between seed wave and pump wave is controlled manually by a mirror attached to a piezoelectric actuator, deamplified amplitude-

squeezed light, amplified phase-squeezed light and light squeezed in an arbitrary quadrature are easily generated. The coherent excitation of the sidebands is controlled coarsely by changing the power of the seed wave (the photon flux of the carrier E_0 at the OPA output port is about 6×10^8 photons $s^{-1} = 120$ pW), fine control is achieved by varying the modulation strength of the EOM. By turning the modulation off, we obtain squeezed vacuum, which has been described in detail previously^{13,14}. By blocking the OPA pump wave, we are left with coherent states.

The signal is analysed at a homodyne detector, whose output current i_- is mixed with an electrical local oscillator $\sim \sin(\Omega t + \phi)$ phase-locked to the modulation frequency, and then low-pass filtered with 100 kHz bandwidth. We fix the phase of the electric local oscillator to $\cos \phi = 1$, so that the resulting current is;

$$i_{\Omega}(\theta, t) = (2\beta E_0 + X_n(\Omega, t) - X_n(-\Omega, t)) \sin \theta + (Y_n(\Omega, t) - Y_n(-\Omega, t)) \cos \theta \quad (2)$$

where $X_n(\Omega, t)$, $Y_n(\Omega, t)$ are the noise fluctuations in a 100-kHz-wide band centred at Ω . By variation of the optical local-oscillator phase θ , any quadrature of the field difference at $\omega + \Omega$ and $\omega - \Omega$ can be accessed.

The i_{Ω} data (about 500,000 points per trace) are taken with a high-speed 12 bit analogue-to-digital converter, while the local-oscillator phase is swept by 2π in approximately 200 ms. Time traces of i_{Ω} for coherent and squeezed states are shown in the left column of Fig. 2. They can be considered to be the experimental counterpart of the theoretical depictions of squeezed states introduced by Caves³⁰.

The traces are subdivided into 128 equal-duration intervals within which the local-oscillator phase is approximately constant. These individual time traces may be regarded as the quantum trajectories of a particular quadrature X_{θ} . The specific behaviour of the trajectory is unpredictable; its statistics however contain the information necessary and sufficient to calculate the quantum state properties. Experimentally, the statistics are obtained by forming histograms of 256 amplitude bins for each quantum trajectory and normalizing the absolute bin width using as reference the distribution of a vacuum state. The middle column of Fig. 2 shows selected measured quadrature probability distributions for the generated states. All distributions are found to be gaussian. This is expected, as the states are generated from a coherent state with a gaussian Wigner function via a second-order nonlinear interaction.

The variances of these distributions determine the amount of squeezing and anti-squeezing. A maximum of -6 ± 0.25 dB

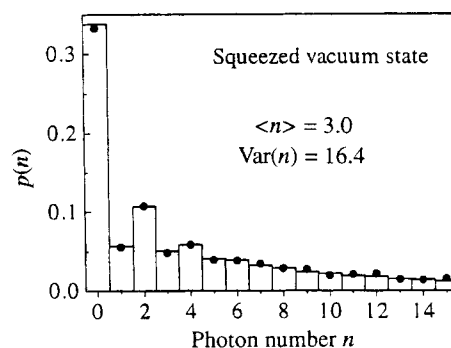
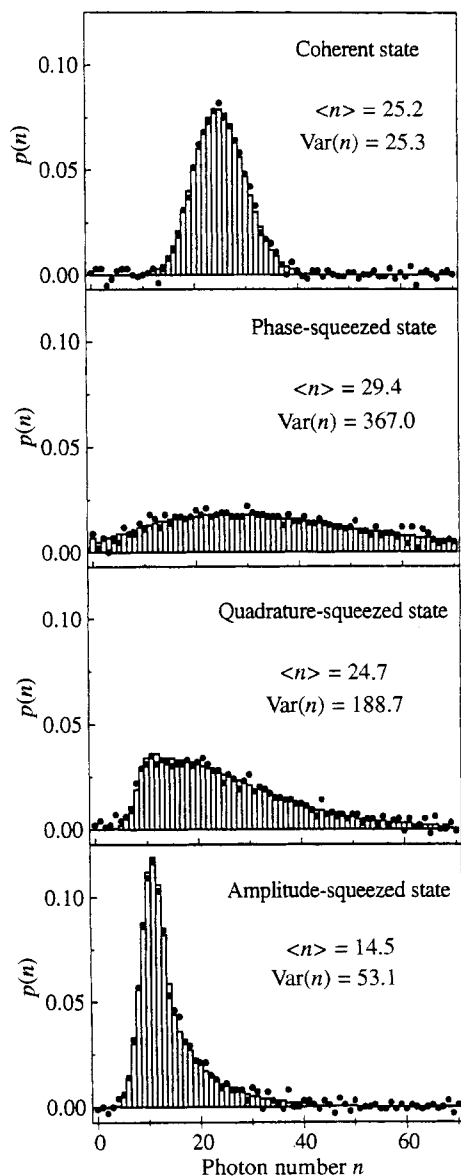


Figure 3 Photon number distributions for the states of Fig. 2. Solid points refer to experimental data, histograms to theoretical expectations. Except for the poissonian distribution of the coherent state, all distributions are super-Poissonian ($\langle n \rangle < \text{Var}(n)$). The odd/even oscillations in the photon number distribution of the squeezed vacuum state are a consequence of the pair-wise generation of photons. They can also be explained by quantum interference effects in phase space³⁷.

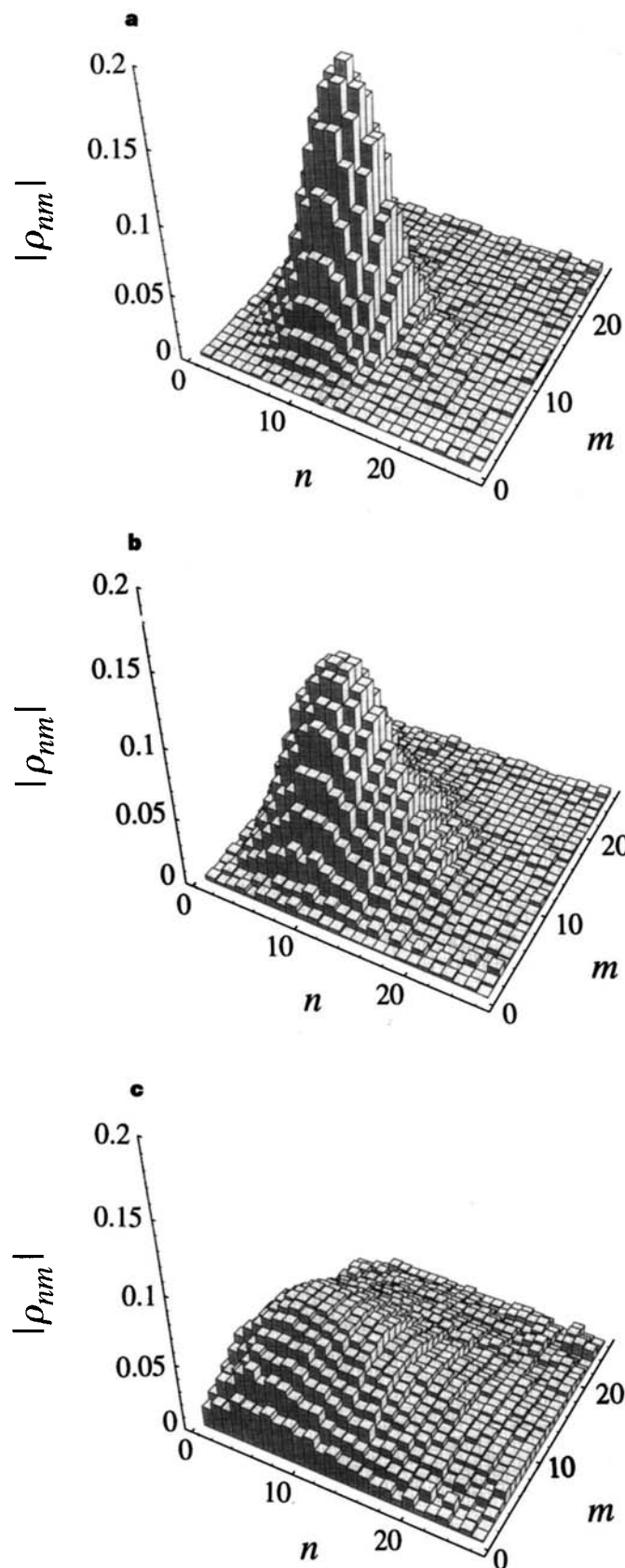


Figure 4 Reconstructed density matrices (absolute values) of three states with approximately equal amplitude: **a**, sub-poissonian amplitude-squeezed state with $\langle n \rangle = 8.9$, $\text{Var}(n) = 4.9$; **b**, coherent state with $\langle n \rangle = 8.4$, $\text{Var}(n) = 8.6$; **c**, phase-squeezed state with $\langle n \rangle = 8.4$, $\text{Var}(n) = 24.6$. The bump around $n \approx 17$, $m \approx 12$ for the amplitude-squeezed state is a characteristic feature, where the matrix elements change sign.

(= 0.25) for the squeezed vacuum mode was detected. For the bright squeezed light only a maximum amount of squeezing of -5.2 dB (= 0.3) was reached, due to slight phase instabilities of the seed wave. The anti-squeezing amounted to 12–14 dB (= 15.8–26.9) for the states presented. These values agree with the results of simultaneous measurements with a spectrum analyser.

Phase-space distributions of squeezed states

Applying the inverse Radon transform yields the Wigner distributions shown in the right column of Fig. 2. They agree well with the theoretical expression;

$$W(x, y) = \frac{1}{\pi ab} \exp \left(-\frac{(x - e_0 \cos \phi)^2}{a^2} - \frac{(y - e_0 \sin \phi)^2}{b^2} \right) \quad (3)$$

where $x = x_0 \cos \phi + x_{\pi/2} \sin \phi$, $y = -x_0 \sin \phi + x_{\pi/2} \cos \phi$ are the phase-space coordinates used in standard textbooks, a and b are respectively the minimum and maximum standard deviation of the quadrature fluctuations, and $e_0 = 2\beta E_0$ is the state's amplitude. The commonly used depiction of squeezed states as ellipses in phase space, with half-axes a and b , corresponds to a horizontal section through the Wigner function. The area of the ellipse is a measure of the purity of the state, as $\text{Tr} \rho^2 = 2\pi \iint W(x, y)^2 dx dy$ equals $1/ab$ for squeezed states (here Tr indicates the trace of a matrix). $\text{Tr} \rho^2$ amounted to 1 for the (pure) coherent state and 0.41–0.46 for the squeezed states of Fig. 2. Their significantly mixed character arises mostly from loss experienced in the cavity of the OPA (escape efficiency 0.88) and during propagation and detection (overall detection efficiency $\eta = 0.94$).

The quantum efficiency of the detection system is a critical issue in the field of QSR³¹. The loss suffered by the quantum state in propagation and detection is equivalent to a convolution of its original Wigner function with a gaussian. Thus for a given detection efficiency η only the s -parametrized phase-space distribution function $W(x, y, s)$ (ref. 18) with $s < 1 - (1/\eta)$, can be reconstructed³². In a strict sense the Wigner function itself $W(x, y) = W(x, y, s = 0)$ is not accessible by tomographical methods, but with our high detection efficiency our reconstructions yield phase-space distributions with $s = -0.064$, which is very close to the Wigner function. An additional smoothing, also a convolution with a gaussian, occurs within the reconstruction algorithm in a filtering procedure with a quadratic regularization method³³. However, its contribution to the total s -parameter can be made less than -0.01 .

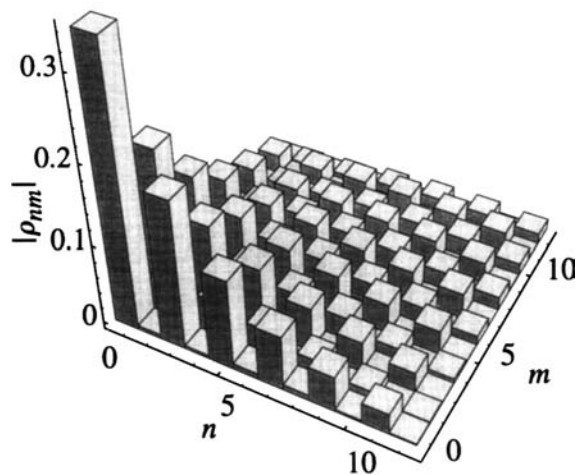


Figure 5 Reconstructed density matrix of the squeezed vacuum state of Fig. 2: along the diagonal and the near off-diagonals the elements alternate in magnitude, which can be explained by quantum interference in phase space³⁷. Odd off-diagonals are zero, owing to the symmetry of the state's distribution in phase space, $W(x, y) = W(-x, -y)$.

Density matrices of squeezed states

An alternative view of the generated states is provided by their density matrices ρ in the Fock basis, because here the state is described in terms of energy eigenstates, in contrast to the description by field components discussed in the previous paragraph. The diagonal elements of the density matrix $\rho_{nn} = p(n)$, are the occupation probabilities of the number states $|n\rangle$. Here n is to be interpreted as the photon flux per unit bandwidth; $p(n)$ is the probability that an ideal photon counter would register in 1 second n photons in a 1-Hz-wide spectral band. A state with $\langle n \rangle$ photons corresponds to a photon flux of $\langle n \rangle \times 10^5$ photons $s^{-1} \approx \langle n \rangle 0.02$ pW in the 100-kHz-wide spectral bands centred at $\omega \pm \Omega$.

Figure 3 shows the photon number distributions for the states from Fig. 2. As can be seen, a simple rotation of the squeezing ellipse (with respect to the coherent excitation in phase space) changes the photon distribution function substantially. Apart from the poissonian distribution of the coherent state, all distributions shown are strongly super-poissonian, that is, the photon number variance $\text{Var}(n)$ exceeds its mean $\langle n \rangle$. For amplitude-squeezed light this seems counterintuitive, as reduced amplitude noise should imply reduced intensity (photon number) noise. An explanation is given by the expressions for photon number average and variance for general (non-minimum-uncertainty) squeezed states³⁴:

$$\begin{aligned} \langle n \rangle &= \frac{1}{4}(a^2 + b^2 - 2) + \frac{1}{2}e_0^2 \\ \text{Var}(n) &= \frac{1}{8}(a^4 + b^4 - 2) + \frac{1}{2}e_0^2(a^2 \cos^2 \phi + b^2 \sin^2 \phi) \end{aligned} \quad (4)$$

For states with a large amplitude e_0 , the variance of the amplitude quadrature $a^2 \cos^2 \phi + b^2 \sin^2 \phi$ indeed determines the characteristics of the photon number distribution. However, in the regime of low amplitudes, when coherent excitation and quantum noise are comparable in size, the first terms in equation (4), figuratively the photon content of the quadrature fluctuations, play a significant role. We adjusted the experimental parameters to $a^2 = 0.43$, $b^2 = 3.3$ (reduced squeezing and anti-squeezing) and $e_0 = 4.12$ to obtain amplitude-squeezed sub-poissonian light. Its Mandel-Q-parameter $(\text{Var}(n) - \langle n \rangle)/\langle n \rangle = -0.45$ is to our knowledge the lowest value achieved so far using optical nonlinear frequency-conversion techniques³⁵.

Figure 4 shows the density matrix up to $n, m = 25$ for the sub-poissonian amplitude-squeezed state in comparison with those of a coherent and super-poissonian phase-squeezed state with approximately equal average photon numbers. Owing to their reflection symmetry in phase space, it is always possible to choose a basis in which the density matrices of these three states in the Fock representation are real. For the coherent state and the phase-squeezed state all elements ρ_{nm} are positive, for the amplitude-squeezed state the near-diagonals show oscillations. The density matrix of the squeezed vacuum, Fig. 5, exhibits the most interesting structure. Its typical 'chess-board' pattern is due to the down-conversion process occurring in the OPA, where photons are created in pairs. The deviations of the experimental density matrices presented here from the theoretical ones are of the order of 0.01 per element. Besides statistical effects, this is partly due to instabilities of the relative phases θ and ϕ and to fluctuations in the pump power.

We have carried out a complete experimental characterization of the whole family of squeezed states. Average photon number and orientation of the states in phase space were accurately controlled by macroscopic experimental parameters. In particular, this flexibility allowed us to generate amplitude-squeezed light with either sub- or super-poissonian photon statistics. The quantum state reconstructions were performed in quasi real-time, with a data acquisition time of 200 ms and an analysing time of ~ 20 s. Our results are in very good agreement with theory. Beyond the reconstructions

presented here, we have investigated the Pegg–Barnett phase distribution and incoherent superpositions of coherent states³⁶.

Quantum state reconstruction by homodyne tomography has been developed into a reliable and accurate tool. We believe that this powerful method will stimulate experimental efforts to generate new quantum states with non-gaussian statistics using higher-order nonlinear processes. $\square$

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The optical counterpart to γ -ray burst GRB970228 observed using the Hubble Space Telescope

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Although more than 2,000 astronomical γ -ray bursts (GRBs) have been detected, and numerous models proposed to explain their occurrence¹, they have remained enigmatic owing to the lack of an obvious counterpart at other wavelengths^{2–5}. The recent ground-based detection^{6,7} of a transient optical source in the vicinity of GRB970228 (refs 8–11) may therefore have provided a breakthrough. The optical counterpart appears to be embedded in an extended source which, if a galaxy as has been suggested^{7,12}, would lend weight to those models that place GRBs at cosmological distances. Here we report observations using the Hubble Space Telescope of the transient counterpart and extended source 26 and 39 days after the initial γ -ray outburst. We find that the counterpart has faded since the initial detection (and continues to fade), but the extended source exhibits no significant change in brightness between the two dates of the observations reported here. The size and apparent constancy of the extended source imply that it is extragalactic, but its faintness makes a definitive statement about its nature difficult. Nevertheless, the decay profile of the transient source is consistent with a popular impulsive-fireball model¹³, which assumes a merger between two neutron stars in a distant galaxy.

The optical counterpart to GRB970228 was observed with the HST Wide Field and Planetary Camera (WFPC2) on 26 March¹⁴ and 7 April¹⁵ 1997, about 26 and 39 days after the outburst, respectively. The optical counterpart was placed at the centre of the Planetary Camera field of view, so as to attain maximum spatial resolution. Three other candidates (one radio¹⁶ and two optical^{17–22}) proposed earlier as counterparts of GRB970228 are also within the WFPC2 field of view. Observations were taken using the F606W (wide V) and F814W (I) filters²³. During each observing run, four exposures with a total integration time of 4,700 s were taken in the F606W filter and two exposures with a total integration time of 2,400 s were taken in the F814W filter. The images were corrected for bias and flat-field variations through the standard Space Telescope processing software. The number of images was sufficiently large to allow for a proper cosmic-ray rejection from the flat-fielded images. The images were combined after cosmic ray rejection. Figure 1a shows a part of the combined F606W and F814W images (for the two observations) where the optical counterpart of the GRB can be seen at the centre. The optical counterpart is embedded in an extended source which can be seen in both passbands. An examination of the point-spread function of different sources also shows

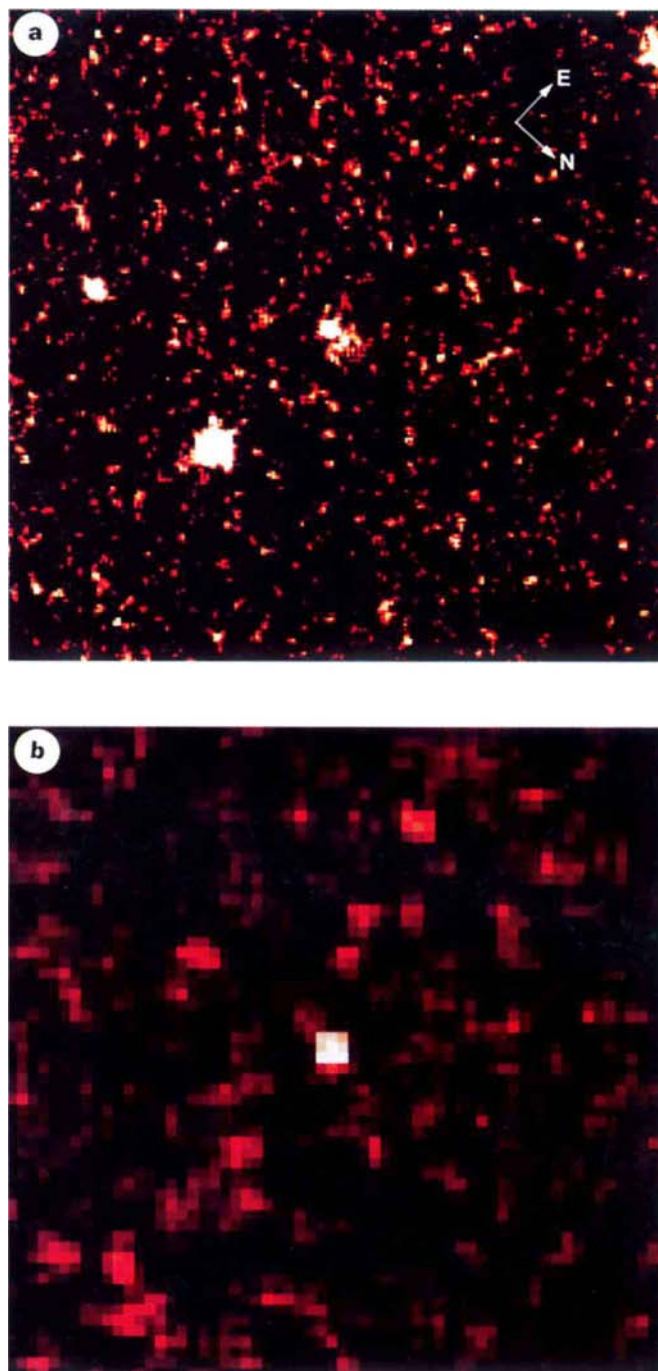


Figure 1 The optical counterpart to GRB970228 was observed with the HST Wide Field and Planetary Camera (WFPC2), between March 26.33 and 26.49 ut, about 26 days after the outburst²⁴, and again between April 7.15 and 7.32 ut, 39 days after the outburst¹⁵. **a**, The sum of F606W and F814W images (resolution 0.045 arcseconds per pixel) taken at both epochs smoothed with a 3×3 spatial filter. The directions of north and east are shown by arrows and the size of the image is 11.5×11.5 arcsec. The point source is at the centre of this image; it is embedded in a diffuse source, 0.3 arcsec to the north. **b**, Difference image obtained by subtracting the summed image of 7 April from the summed image of 26 March (amplified 4 times with respect to **a**). The point source is clearly seen, but there is no trace of the extended source. This is consistent with fading of the point source and constancy of the extended source (within errors) between the two observations. Black represents the lowest intensity, white represents the highest intensity and red is intermediate. The intensity of the extended component is well above the noise level (as seen in the individual pixels throughout the image) in both filters and in both epochs of observations.

clearly the extended nature of this source. As seen in Fig. 1a, the angular extent of the extended source is ~ 1 arcsec and it is elongated in the east–west direction. The point source lies ~ 0.3 arcsec south of the centre of the extended source. Photometry in both wavebands was carried out for the point source using the photometric software package DAOPHOT. The derived magnitudes for the point source are given in Table 1. As noted earlier, this source was first detected in the optical wavelengths about 1 day after the outburst, when the magnitudes were $V = 21.3$ and $I = 20.6$ and the magnitudes observed 9 days after the outburst were $V > 23.6$ and $I > 22.2$. The observed decline in brightness is shown in Fig. 2 with the predictions of various models (see discussion below). The $V-I$ colour of the point source has increased by about 0.6 mag in 25 days, which is consistent with a cooling trend, and then remained roughly constant for the following 12 days.

We tested for a possible proper motion of the point source between the two epochs 26 March and 7 April in the two filters. We find, both in the V and I filters, a potential motion in the NW direction of ~ 0.006 arcsec. Given the errors involved (this represents a 1.5 to 2σ result), and considering the fact that the GRB is the faintest object in the field and is embedded in a nebosity, we conclude that no proper motion has been detected.

We point out that, even without discussing the probability of finding a variable star in the error box of the GRB during the given time frame, we can rule out the possibility that the optical transient is actually an unrelated nova, dwarf nova, flare star or a supernova. The evolution of the colours is inconsistent with that of novae (the transient becoming redder rather than bluer following maximum)²⁴, and the value of $(V-I)$ (which is ~ 0.0 for dwarf novae) and its development are inconsistent with those of dwarf novae²⁵ (correction for absorption indicated by the (0.1–10 keV) observations on board the SAX satellite does not change this conclusion). The duration of the event is longer by orders of

magnitude than those of stellar flares²⁶. Although supernovae decay in visual wavelengths at a slower rate than observed for the optical transient, they can decay in the ultraviolet as fast as 2.5 mag per day (ref. 27). Thus, a supernova at the appropriate redshift ($z \approx 1-2$) could appear to decay in the visual (ultraviolet rest frame) at the observed rate. However, the observed fast decline in I would imply that the redshift of this supernova is > 1 . Such a large redshift would make this object brighter than the brightest supernovae (observed either at low²⁸, or at high^{29,30} redshift) by about 2 mag at the peak, thus making it improbable that it is a supernova.

To determine the magnitudes for the extended source, the point source was subtracted using a point-spread function derived from other point sources in the images. DAOPHOT was used to derive the magnitudes of the extended source within a radius of 0.6 arcsec; the resultant magnitudes are given in Table 1. The angular dimensions of the extended source are consistent with earlier estimates from the ground; however, its brightness appears lower by ~ 0.9 mag in the 26 March observation. At present it is not entirely clear whether this discrepancy represents a real decline, or whether it simply results from the inability of the ground-based observations to resolve the point and extended sources. To investigate this further, we performed an experiment to simulate the ground-based observations of 9 March. We used the combined F606W image and artificially increased the brightness of the point source to $V = 24.0$ (as predicted by the best theoretical models, see below). We then smoothed the image to simulate 1-arcsec seeing from the ground. In this simulated image (not shown) the extended nature of the source is barely discernible and the brightness of the individual components cannot be separately determined. We note that visual comparison of the images of the extended source taken on 26 March and 7 April reveals small differences. Taken at face value, these differences could be interpreted as indicating changes in either the relative brightness of different parts of the extended source, or in its position. However, no such definitive statement can be made, given the errors involved in the fluxes. This conclusion is further strengthened by the following exercise. We subtracted the image of 7 April from that of 26 March, and the result is shown in Fig. 1b. The point source is clearly visible in the subtracted image (which is consistent with its decline in brightness), while no trace of the extended source may be distinguished from the background noise. Thus, within the errors, it appears that the extended source (which dominates the light after 13 March) remained constant.

If the extended source was indeed declining (or changing in position), the implication would have been that it is probably powered by the GRB. As the angular dimensions of the source are ~ 1 arcsec, a brightness variation in 15 days would imply a distance to the source of no more than ~ 2.7 kpc (assuming that the light comes from the source location, and is not scattered from an intervening 'screen'). Such a short distance would not be consistent even with models which place GRBs in an extended Galactic halo³¹, as these models require typically distances significantly larger than the distance between Earth and Galactic centre. Models placing all the GRBs in the Galactic disk have been convincingly ruled out by observations³². Clearly, with only one optical counterpart to date, we cannot rule out the possibility of the existence of two populations of GRBs^{33,34}, one at cosmological distances and the other of Galactic origin. The above discussion does suggest that the extended source remained constant and that the apparent decline in brightness is merely a consequence of the low resolution of the ground-based observations. If this interpretation is correct, then the extended source could be an external galaxy (its colour is inconsistent with it being Galactic cirrus as seen in the infrared observations of IRAS). We also note that the observations of SAX LECS indicate an absorption towards the source of $A_V \approx 2.5$ mag. With the foreground extinction being³⁵ $A_V = 0.4 \pm 0.3$, this could indicate absorption in a galaxy (although the possibility of circumstellar absorption in the vicinity of the source cannot be ruled out).

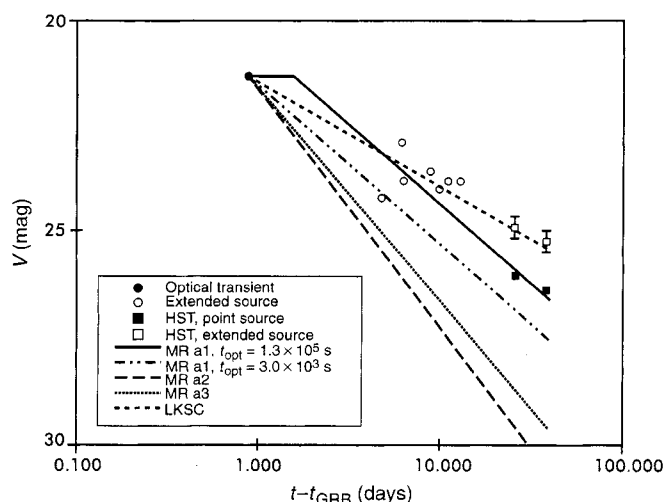


Figure 2 Measured brightness of the GRB970228 optical counterpart and models of γ -ray burst remnants. The brightness of the optical counterpart has been measured by Groot *et al.* in the 4.2-m William Herschel Telescope (WHT) discovery image^{6,7} (filled circle, labelled "Optical transient"). The HST measurements presented in this Letter are indicated by filled squares (point source) and empty squares (adjacent extended source). The measured brightness^{6,7,2,44} of an extended source at the position of the transient is indicated by empty circles. The Meszaros and Rees models¹³ for the brightness of GRB remnants are labelled in the key as "MR". In model a1, only the forward blast wave radiates efficiently; in model a2, both the forward and the reverse shocks are efficient radiators; model a3 is similar to a2, but has a different origin for the magnetic field. The Liang *et al.* model³⁸ is labelled in the key as "LKSC". ($t - t_{\text{GRB}}$ is the time elapsed since the burst; t_{opt} is the time at which the peak frequency drops below the optical band.)

Table 1 HST photometry of GRB970228 field

Source	Date (UT)	V (mag)	I (mag)
Point source	Mar. 26.4	26.1 ± 0.1	24.2 ± 0.1
Point source	Apr. 7.2	26.4 ± 0.1	24.6 ± 0.1
Ext. source	Mar. 26.4	24.9 ± 0.3	24.5 ± 0.3
Ext. source	Apr. 7.2	25.2 ± 0.35	24.3 ± 0.35

The magnitudes given here are in the Cousins bands, where the magnitudes have been transformed from WFPC2 to Cousins filters taking an appropriate colour correction into account. For colour correction, an M2 type spectrum is assumed for the point source and an F2 type spectrum is assumed for the extended source. As Cousins I filter is similar to F814W, the colour correction is close to zero in the I band.

As we have found that the optical counterpart may be associated with a galaxy, we first have to determine the probability of a chance superposition. The latter can be estimated by the fractional area covered by galaxies of 25th magnitude (in V), or brighter. From the catalogue computed from the Hubble Deep Field³⁶ using the software package FOCAS, we derive a probability for chance superposition of the order of 4%. This is consistent with the probability estimates of van Paradijs *et al.*⁷

In the following we will therefore assume that the GRB is located in an external galaxy and we will examine the implications of this assumption for theoretical models of GRBs.

One of the key components that can lead to a better understanding of GRBs is the question of their location. The isotropy and inhomogeneity in the distribution of the bursts have led to two leading classes of models, one placing the sources at cosmological distances³², and the other placing them in the extended galactic halo³¹. The association of GRB970228 with an external galaxy would clearly support the cosmological models.

A second question that can be addressed is whether GRBs are associated with nuclear activity of active galactic nuclei (for example, the burst being produced by tidal disruption of a star³⁷). The HST observations clearly show that the optical counterpart is not located at the centre of the brightness distribution, suggesting that GRB970228 is not at the centre of the host 'galaxy'. Although inconclusive, this suggests that GRBs as a class are not related to central supermassive black holes.

Irrespective of the nature of the extended source (but assuming that the optical transient point source is indeed the GRB), the data provide valuable information on the evolution of GRB remnants. In particular, specific predictions for the temporal behaviour of the optical emission from cooling and expanding fireball ejecta (in the context of cosmological models) were made by Meszaros and Rees¹³ and (for X-rays in the context of Galactic halo models) by Liang *et al.*³⁸. In Fig. 2, we show the predictions of the impulsive-fireball models of the former authors and of the model by the latter authors, in which cooling of the non-thermal leptons occurs by saturated Compton upscattering, together with the optical observations. As seen in Fig. 2, our results are consistent with a simple impulsive model¹³ in which only the forward blast wave radiates efficiently and in which the peak frequency drops below the optical band about 1.7 days after the burst ($t_{\text{opt}} \approx 1.7$ days). It is important to note that the observations rule out models¹³ in which the energy input is continuous rather than impulsive, in their simplest form. These models predict either no optical flux at all after the γ -ray flash, or a flux which declines as t^{-6} , much faster than observed. We also note that the blast wave models predict a change in their power law (towards a steeper decline) after the blast wave has 'snowploughed' through a rest-mass energy of the order of the burst energy. This predicts a change in the power law after a few days, for a burst energy of 10^{42} erg (corresponding to extended Galactic halo models). No such change has been observed. If anything, a change in the power law to a shallower decline may have been detected³⁹ on 6 March. This tends to support a cosmological origin for the burst (in which case a change is expected only after a timescale of years).

The fact that cosmological models require peak luminosities of

the order of $10^{51} \text{ erg s}^{-1}$ has led to the popularity of models involving the mergers of either two neutron stars, or a neutron star and a black hole^{40,41}. The frequencies of such mergers have been estimated by Phinney⁴² and by Narayan, Piran and Shemi⁴³. Using the 'best guess' values for the parameters from Phinney and a Hubble constant of $H_0 = 65 \text{ km s}^{-1} \text{ Mpc}^{-1}$, we obtain a value of ~ 80 for the ratio of the neutron-star merger rate in disk galaxies to that in ellipticals. Thus if GRBs are produced by such mergers, then binaries in disks should dominate the observed rate. A close examination of the 'host galaxy' of GRB970228 (Fig. 1a) indeed reveals elongated features to the east, which are suggestive of a spiral or irregular morphology, rather than an elliptical one. Furthermore, the $V-I$ colour of the extended source (admittedly uncertain) suggests a late-type galaxy. A definitive answer to the nature of the extended source associated with GRB970228 should be provided by HST observations after a longer time interval. $\square$

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The decay of optical emission from the γ -ray burst GRB970228

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The origin of γ -ray bursts has been one of the great unsolved mysteries in high-energy astrophysics for almost 30 years. The recent discovery of fading sources at X-ray¹ and optical^{2,3} wavelengths coincident with the location of the γ -ray burst GRB970228 therefore provides an unprecedented opportunity to probe the nature of these high-energy events. The optical counterpart appears to be a transient point source embedded in a region of extended nebulosity³⁻⁶, the latter having been tentatively identified as a high-redshift galaxy³. This would seem to favour models that place γ -ray bursts at cosmological distances, although a range of mechanisms for producing the bursts is still allowed. A crucial piece of information for distinguishing between such models is how the brightness of the optical counterpart evolves with time. Here we re-evaluate the existing photometry of the optical counterpart of GRB970228 to construct an optical light curve for the transient event. We find that between 21 hours and six days after the burst, the R-band brightness decreased by a factor of ~ 40 , with any subsequent decrease in brightness occurring at a much slower rate. As the point source faded, it also became redder. The initial behaviour of the source appears to be consistent with the 'fireball' model⁷, but the subsequent decrease in the rate of fading may prove harder to explain.

The γ -ray burst of 28 February 1997, detected⁸ with the Gamma-Ray Burst Monitor on board the BeppoSAX satellite, and located with an $\sim 3'$ radius position with the Wide Field Camera on the same satellite, was the first for which a fading X-ray¹ and optical counterpart^{2,3} were discovered.

The optical counterpart was discovered^{2,3} from a comparison of V- and I-band images taken with the William Herschel Telescope (WHT) on February 28.99 UT, and the Isaac Newton Telescope (INT; V band) and the WHT (I band) on March 8.8 UT. After the counterpart had weakened by several magnitudes, it was found to coincide with an extended object^{9,10}. In subsequent observations with the Hubble Space Telescope (HST) on 26 March and 7 April 1997, Sahu *et al.*⁴⁻⁶ found that the optical counterpart consists of a

point source and an extended ($\sim 1''$) object, offset from the point source by $\sim 0.5''$. The extension is in the same direction as that seen in the earlier ground-based observations.

In view of the importance of the optical light curve in constraining possible models of γ -ray bursts, we have re-assessed the photometric information presented by Van Paradijs *et al.*³, together with more recent information in light of the HST findings, and derived an optical light curve of the point source component of the GRB counterpart.

In Table 1 we have collected the available optical photometry on GRB970228. These results have been obtained in several passbands, primarily V, R and I (effective wavelengths $\sim 5,500 \text{ \AA}$, $\sim 6,500 \text{ \AA}$ and $\sim 8,000 \text{ \AA}$, respectively, corresponding closely to the Cousins VRI system). As the R band occupies a central position relative to most of the passbands used in the available observations, we have reduced all photometry to the R band. In the required interpolations we have assumed that the spectra of the point source and the extended emission are similar to main-sequence stars (that is, not dominated by emission lines). We have used the relation between the colour indices $V - R$ and $V - I$ given by Thé *et al.*¹¹ for late-type stars; for bluer stars we have inferred this relation from the tables given by Johnson¹² for main-sequence stars and the colour transformations to the Cousins VRI system given by Bessell¹³. For given magnitudes V and I the uncertainty in the interpolated magnitude R is determined by the uncertainty in the ratio $\alpha = (V - R)/(V - I)$, because $R = V - \alpha(V - I)$. Over the range in $(V - I)$ covered by the point source, this ratio α varies between 0.45 (at $V - I = 0.7$) and 0.55 (at $V - I = 2.0$). We have made alternative estimates of α from numerical integrations of power-law flux distributions ($F(\lambda) \propto \lambda^\beta$, with β ranging between -4 and $+2$), and of Planck functions (with temperatures between 3,000 and 10,000 K), each multiplied by V, R and I response functions¹⁴. We find that these 'theoretical' α values cluster near 0.4, that is, only slightly smaller than the values observed for stars. We conclude that if the flux distribution of the optical counterpart is smooth, the uncertainty in the interpolation of R from V and I is unlikely to exceed 0.1 mag.

From the V and I magnitudes obtained from the two HST images (Table 1; to arrive at these values we applied a colour-dependent correction term¹⁵) we infer that between the two HST observations the point source became marginally fainter; the extended source did not show a significant brightness change.

In the 28 February observation with the WHT the optical transient is much brighter than any steady counterpart, and the

Table 1 Summary of optical observations

Date	Time (UT)	Telescope	Magnitude	Remarks
Feb. 28	18.4	BUT	$R = 21.1 \pm 0.2$	OT
Feb. 28	19.4	RAO	$R = 20.6 \pm 0.5$	OT
Feb. 28	23.8	WHT	$V = 21.3 \pm 0.1$	OT
Feb. 28	23.8	WHT	$I = 20.6 \pm 0.1$	OT
Mar. 03	2.4	AP0	$B = 23.3 \pm 0.5$	OT
Mar. 04	20.7	NOT	$V > 24.2$	OT + ext. source
Mar. 06	7.7	Keck	$R = 24.0$	OT + ext. source
Mar. 08	20.7	INT	$V > 23.6$	OT + ext. source
Mar. 08	21.2	WHT	$I > 22.2$	OT + ext. source
Mar. 09	21.5	INT	$R = 24.0 \pm 0.2$	OT + ext. source
Mar. 09	20.5	INT	$B = 25.4 \pm 0.4$	OT + ext. source
Mar. 13	0.0	NTT	$R = 24.3 \pm 0.2$	OT + ext. source
Mar. 26	9.2	HST	$V = 26.1 \pm 0.1$	OT
Mar. 26	11.2	HST	$I = 24.2 \pm 0.1$	OT
Mar. 26	9.2	HST	$V = 24.9 \pm 0.3$	Ext. source
Mar. 26	11.2	HST	$I = 24.6 \pm 0.3$	Ext. source
Apr. 7	5.2	HST	$V = 26.4 \pm 0.1$	OT
Apr. 7	7.2	HST	$I = 26.4 \pm 0.1$	OT
Apr. 7	5.2	HST	$V = 25.2 \pm 0.35$	Ext. source
Apr. 7	7.2	HST	$I = 24.3 \pm 0.35$	Ext. source

Abbreviations: OT, optical transient; ext. source, extended source; BUT, Bologna University Telescope; RAO, Rome Astrophysical Observatory; WHT, William Herschel Telescope; AP0, Apache Point Observatory; NOT, Nordic Optical Telescope; INT, Isaac Newton Telescope; HST, Hubble Space Telescope.

latter's contribution can therefore be entirely neglected. From a re-analysis of the images we confirm the (V, I) magnitudes for the transient as given before^{2,3} (Table 1), but those for the substantially weaker nearby star are somewhat different: (V, I) = (23.0, 21.2). The latter values are in good agreement with those obtained with the HST: (V, I) = (23.0, 21.4).

Guarnieri *et al.*¹⁶ found no variable objects in B- and R-band images taken with the Bologna University Telescope on February 28.76, and on March 1.81, 3.76, 4.82 and 5.86 UT. However, a subsequent analysis of these images confirms that the optical transient is present on February 28.76 UT at the position given by Groot *et al.*⁹ with $R = 21.1 \pm 0.2$ (A. Guarnieri, personal communication).

Pedichini *et al.*¹⁷ confirmed the detection of the optical transient on a Schmidt CCD image taken at the Rome Astrophysical Observatory on February 28.81 UT; it was then 1.6 ± 0.5 mag brighter than the nearby late-type star (for which $R = 22.1$). The broad-band filter used in this observation had a peak at 7,000 Å, similar to that of the R band, but because its width is almost twice that of the latter the inferred $R = 20.5 \pm 0.5$ may suffer from a colour effect. But because on February 28.99 UT (at about the same brightness level) $V - I = 0.7$, this colour effect is likely to be small compared to the quoted error of 0.5 mag.

In an observation with the Apache Point Observatory on March 3.1 UT, Margon *et al.*¹⁸ detected the optical transient at $B = 23.3 \pm 0.5$. We have inferred an estimated R-band magnitude, by interpolating between the colour index $B - R = 1.4$, observed on

9 March (see Table 1), and a value for this colour index on 28 February, $B - R = 1.1$, inferred from the observed $V - I$, assuming again that the ($B - R, V - I$) relation follows that of main-sequence stars. This estimate of R involves an extrapolation rather than an interpolation (as with V and I observations); this entails an additional uncertainty, which we conservatively estimate at 0.5 mag. The corresponding R-band magnitude on March 3.1 UT then is 22.2 ± 0.7 .

In the 4 March observation with the Nordic Optical Telescope, and the 8 March observations with the WHT and the INT, the counterpart was not detected (see Table 1 for the upper limits).

In the 9 March observation with the INT and the 13 March observations with the ESO New Technology Telescope (NTT), the source is detected. In the NTT image the source appears extended; this was found¹⁰ independently from images obtained with the Keck Telescope. The direction of this offset is consistent with being caused by the faint extended emission detected with the HST observations.

We have transformed the collection of V, R and I magnitudes to one common R-band magnitude, in the following way. For near-simultaneous V and I observations we have used the ($V - I, V - R$) relation (see above) to get $R = V - (V - R)$. In case no such simultaneous observations are available, we have interpolated linearly in the relation between ($V, V - I$) obtained from the 28 February and the HST observations. We have collected these results in Table 2.

We note that the R-band magnitude for the combined point source plus extended component obtained from the HST images (Table 2) differs substantially from the value, $R = 24.9 \pm 0.3$, given by Metzger *et al.*¹⁹ on 5 and 6 April 1997. Moreover, their conclusion that the component causing the earlier (ground-based) images to appear extended has faded below detection level seems difficult to reconcile with the HST observation that both in the V and I bands the extended component is at least as bright as the point source.

We have subtracted the contribution of the extended component from the ground-based results. From the corrected HST magnitudes (see above) we infer that the R-band magnitude of the extended component (averaged over the two HST observations) is $R = 24.7 \pm 0.3$. These corrected R-band magnitudes for the point source are also given in Table 2.

The R-band light curve obtained from our analysis of the available photometry is shown in Fig. 1, in which we have plotted R versus $\log t$, where t is the time since the γ -ray burst occurred.

A single power-law decay of the flux (that is, $F_R(t) \propto t^{-\alpha}$) during the entire 38-day period does not represent the data well. The upper limit on R obtained on 4 March and the R magnitude measured on 6 March are incompatible with such decay, which in Fig. 1 would be represented by a straight line.

The initial decline, between 28 February and 6 March was much faster than that after 6 March. Before 6 March the average decay rate was $0.7^{+0.2}_{-0.1}$ mag d⁻¹, during the month following 6 March it was $0.02^{+0.02}_{-0.04}$ mag d⁻¹. Expressed in terms of a power-law exponent α , the average decay before 6 March corresponds to $\alpha = 2.1^{+0.3}_{-0.5}$, whereas after 6 March we find $\alpha < 0.35$. Expressed in terms of an exponential decay time τ of the R-band flux, we find before 6 March $\tau \approx 1.4$ d, and afterwards $\tau > 25$ d.

As the point-source component of the optical transient became fainter, it also became redder, from $V - I = 0.7$ at $V = 21.3$ (28 February) to $V - I = 1.9$ at $V = 26.1$ (26 March), and $V - I = 1.8$ at $V = 26.4$ (7 April).

The exponent of the power law describing the decay of the X-ray afterglow of GRB970228, as obtained from the results given by Costa *et al.*¹ and Yoshida *et al.*²⁰, is $\alpha_X = 1.4 \pm 0.2$. A comparison of this value with the initial power-law slope of the optical brightness decay indicates that during the first week after the γ -ray burst the optical transient decayed more rapidly than the X-ray transient.

From recent comparisons^{6,21} of the optical and X-ray observations of GRB970228 with predictions of the 'fireball' model (see, for

Table 2 The R-band light curve of GRB970228

Date (UT)	Telescope	R (OT + ext. source)	R (OT)
Feb. 28.76	BUT	21.1 ± 0.2	21.1 ± 0.2
Feb. 28.81	RAO	20.5 ± 0.5	20.5 ± 0.5
Feb. 28.99	WHT	20.9 ± 0.14	20.9 ± 0.14
Mar. 03.10	APO	22.2 ± 0.7	$22.3^{+0.9}_{-0.7}$
Mar. 04.86	NOT	>23.3	>23.6
Mar. 06.32	Keck	24.0 ± 0.2	$24.8^{+1.2}_{-0.5}$
Mar. 08.88	INT + WHT	>22.6	>22.8
Mar. 09.90	INT	24.0 ± 0.2	$24.8^{+1.2}_{-0.5}$
Mar. 13.00	NTT	24.3 ± 0.2	$25.6^{+2.6}_{-0.9}$
Mar. 26.42	HST	24.1 ± 0.2	25.17 ± 0.13
Apr. 07.23	HST	24.2 ± 0.15	25.50 ± 0.13

Abbreviations as Table 1.

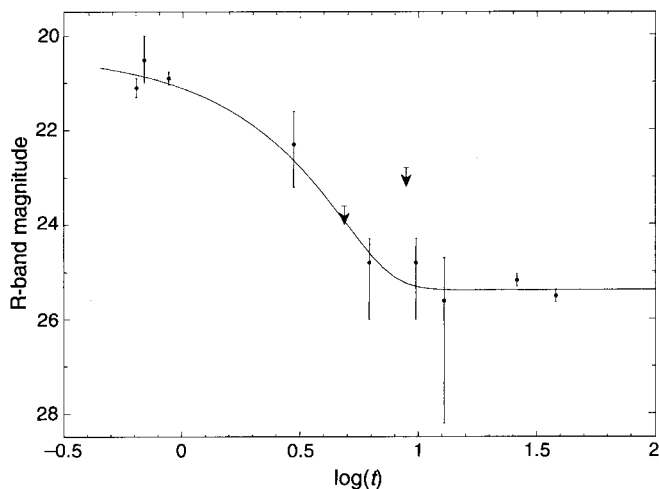


Figure 1 R-band light curve of the point source component in the optical counterpart of GRB970228. The R-band data are from Table 2, and t represents the time interval since the γ -ray burst occurred. For reference, an exponential decay with $\tau = 1.4$ days plus a constant is shown as a solid curve.

example, Meszaros⁷) it has been concluded that this model is consistent with the initial decay of the γ -ray burst afterglow. We note however, that the decay does not continue beyond 6 March. This slow-down of the brightness decrease after 6 March may be the result of changes in one or more factors determining the optical emission, such as changes in source geometry, or optical depth effects. It may signal the changeover to a different dominant energy source. If γ -ray bursts are the result of mergers of neutron star binaries (see, for example, Piran²² and references therein), decay of radioactive nuclei produced by de-neutronization of neutron-star matter²³ may play a role. $\square$

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Pure $d_{x^2-y^2}$ order-parameter symmetry in the tetragonal superconductor $\text{Ti}_2\text{Ba}_2\text{CuO}_{6+\delta}$

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Crucial to the successful development of a theoretical model for high-temperature superconductivity is knowledge of the symmetry of the order parameter (or wavefunction) that describes the pairing of electrons in the superconducting state. Several experimental studies^{1–8} provide convincing evidence for an anisotropic order parameter, consistent with a $d_{x^2-y^2}$ symmetry. But none of these earlier experiments could rule out unambiguously an addi-

tional contribution from isotropic s -wave pairing; these experiments either involved superconductors with an orthorhombic crystal structure (for which a mixed $s+d$ state is becoming increasingly recognized as a likely consequence^{9,10}), or their interpretation required detailed modelling of the uncertain effects of disorder and defects. Here we report the results of an experiment designed to circumvent these difficulties: the material studied is the single-layer tetragonal superconductor $\text{Ti}_2\text{Ba}_2\text{CuO}_{6+\delta}$, and the experimental configuration is such that the interpretation of the results relies solely on symmetry considerations. Our results indicate that this material has pure $d_{x^2-y^2}$ pairing symmetry, so providing a starting point for understanding the more complex mixed $s+d$ state that appears to characterize other high-temperature superconductors.

An $s+d$ pair state in orthorhombic copper oxides such as $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$ (YBCO) is characterized by a gap function $\Delta(\mathbf{k})$ that transforms like $s(k_x^2 + k_y^2) + d(k_x^2 - k_y^2)$, where k_x and k_y are components of the wavevector $\mathbf{k}$, and s and d are measures of the amounts of s -wave and d -wave pairing in the admixture. Node lines in the gap function ($\Delta = 0$) given by $k_y = \pm [(d+s)/(d-s)]^{1/2} k_x$ exist provided that $d/s \geq 1$. The deviation of the slope of the node lines from the diagonals $k_x = \pm k_y$ of the Brillouin zone depends on the extent of the $s+d$ admixture. Because the Pb-YBCO corner superconducting quantum interference device, SQUID, (or single Josephson junction) interference experiments rely on a sign change between the a and b faces of YBCO, they cannot distinguish pure d -wave from mixed $s+d$ pair states as long as $d/s \geq 1$. The tricrystal experiments with YBCO and $\text{Ti}_2\text{Ba}_2\text{CuO}_{6+\delta}$ (Ti2001) can, in principle, locate the nodes on the Fermi surface. However, this requires a systematic series of tricrystal experiments and a detailed model describing pair tunnelling beyond the Sigrist–Rice formula¹¹, which does not take into account interface roughness or disorder.

In the face of these difficulties, it is important to demonstrate unambiguously the existence of a pure $d_{x^2-y^2}$ wave high- T_c copper

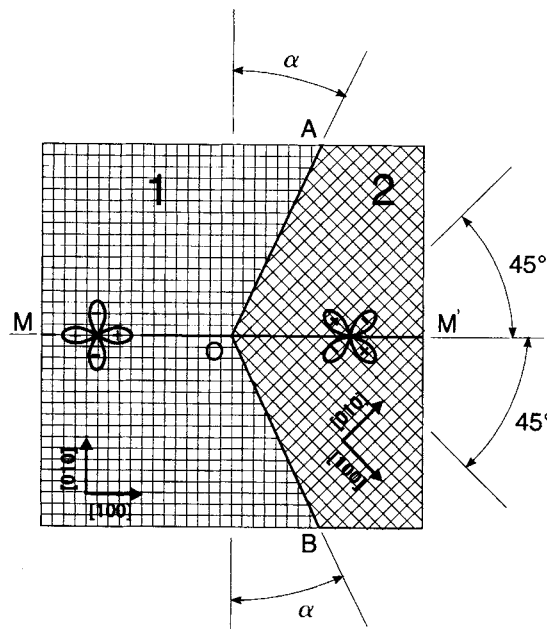


Figure 1 Schematic diagram illustrating the geometrical configuration of a tetracystal (100) SrTiO_3 substrate, which effectively consists of two crystals rotated about the normal to the substrate plane by $\pi/4$ with respect to each other. For a given total misorientation angle $\theta = \theta_1 + \theta_2$, the pair tunnelling current is maximized⁹ for a symmetric grain boundary $\theta_1 = \theta_2 = \pi/8$. We chose the angle $\alpha = \theta_1$ between the vertical ([010] in grain 1) and the grain boundaries (OA and OB) as 25° . Also shown are the polar plots of the assumed $d_{x^2-y^2}$ gap functions aligned with the crystallographic axes in the substrate.

oxide superconductor. Such an experiment would be significant in view of the substantial amount of theoretical work^{12–15} on the effect of $s + d$ wave pairing on the properties of copper oxide superconductors, including the origin of high-temperature superconductivity. The demonstration of a pure d -wave superconductor would help us to understand whether the coexistence of d and s singlet pairing channels is essential or just accidental to high- T_c superconductivity. It would also serve as a well defined starting point for understanding the more complex ($d + s$)-wave superconductors.

Here we use a single-layer tetragonal Tl2201 film deposited on a (100) SrTiO₃ (STO) substrate (manufactured by Shinkosha Co., Tokyo) with a bicrystal geometry proposed by Walker and Luettemer-Strathmann⁹ (Fig. 1). The interpretation of this phase-sensitive test of pairing symmetry in this geometry depends only on symmetry considerations. We achieved the desired bicrystal wedge configuration by effectively fusing two bicrystals along the dividing line MOM' in Fig. 1. There is a very important built-in reflection symmetry with respect to this line. The pair tunnelling current I_s across a tunnel barrier between superconductors 1 and 2 is given by:

$$I_s \propto \langle \Psi_1 | H_T | \Psi_2 \rangle \quad (1)$$

where Ψ_1 and Ψ_2 are the pair wavefunctions in superconductors 1 and 2 respectively, and H_T is the standard generic second-order tunnelling hamiltonian^{16,17}. Assuming that H_T is invariant under

reflection about the line MOM' (Fig. 1), for $d_{x^2-y^2}$ or d_{xy} pairing states a reflection symmetry operation m on I_s results in pair tunnelling across the grain boundary OA that is related to that along OB by:

$$(I_s)_{OB} = m(I_s)_{OA} = -(I_s)_{OA} \quad (2)$$

The same conclusion may be reached by using the general expression for the Josephson current⁹. This sign change between the supercurrents across OA and OB means that any superconducting loop enclosing the wedge tip O will result in a net π phase, which produces a spontaneously generated half flux quantum.

The oxygen-optimized 200-nm-thick c -axis oriented Tl2201 film

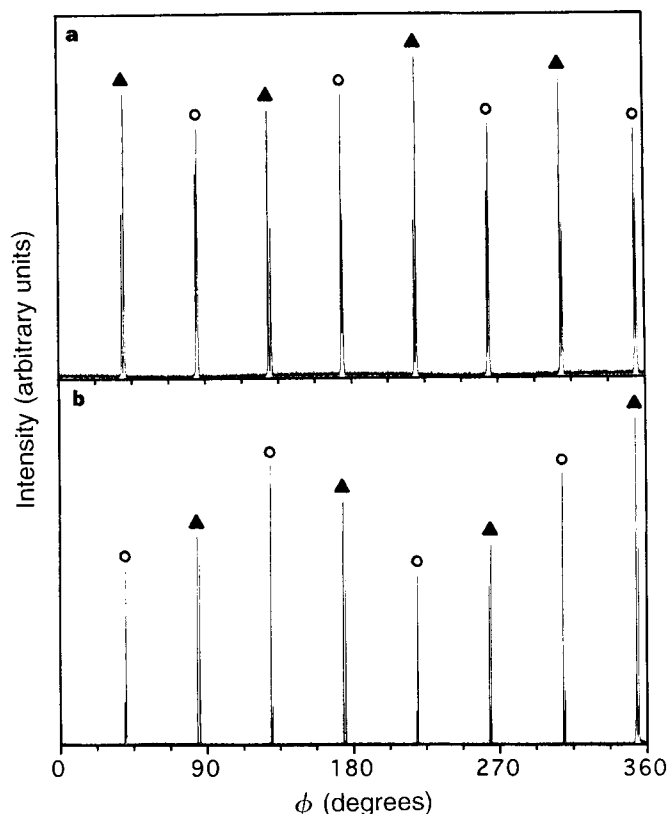


Figure 2 X-ray ϕ scans (0° – 360°) of the (105) reflection from the c -axis oriented epitaxial Tl2201 film (a), and the (111) reflection of the same film's underlying SrTiO₃ substrate (b). Each consists of two sets (labelled with a closed triangle and an open circle) of four 90° -apart peaks. In each case, there is a 45° offset between the two sets of ϕ -scan peaks, confirming the 45° -rotated wedge bicrystal configuration. The angular difference projected onto the basal plane between the (105) and (111) planes leads to the 45° shift in ϕ between the corresponding peaks in a and b. Furthermore, there is a 1° splitting in each of the peaks.

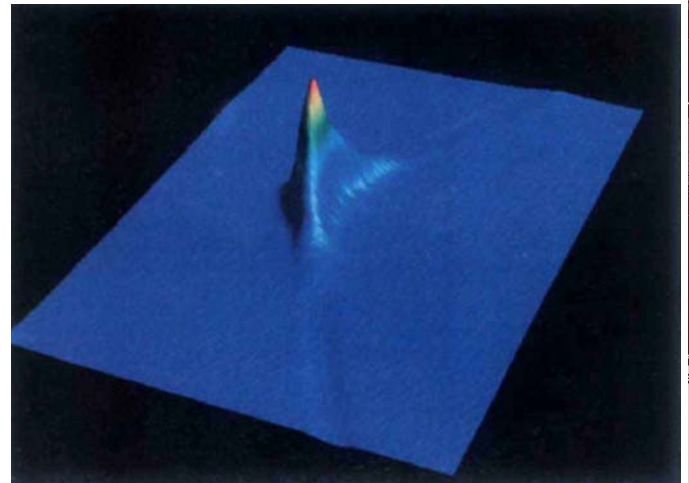


Figure 3 A three-dimensional presentation of the SSM image of the Josephson vortex trapped at the wedge tip in a Tl2201 blanket film deposited on a tetracystal (100) SrTiO₃ substrate of the geometry shown in Fig. 1. The sample was cooled in a field of <1 mG and imaged at 4.2 K.

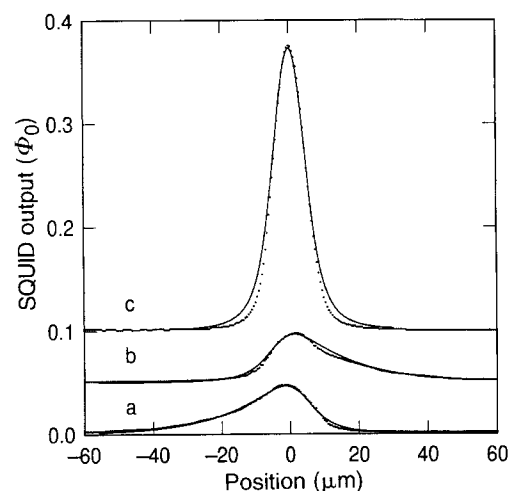


Figure 4 Modelling of the SSM imaging data in Fig. 3 for cross-sections along the directions OA (curve a) OB (curve b), and also through the peak of an isolated bulk single flux quantum in the same Tl2201 film (curve c). The solid lines are fits to the data. The height of the pickup loop was determined to be $5.15 \mu\text{m}$ by fitting the bulk vortex data. If we assume that the total flux is $h/4e$, the best fit to the data (solid curves in a and b) was obtained for a Josephson penetration depth of $12.5 \mu\text{m}$. If we allow both the total flux and the Josephson penetration depth to vary, we find $\Phi/\Phi_0 = 0.54 \pm 0.04$ and $\lambda_J = 13.8 \pm 1.5 \mu\text{m}$, using a criterion of a doubling of the best-fit χ^2 value. The solid line in curve b is derived from that in curve a by mirror reflection about the y -axis. Curves b and c are offset by 0.05 and 0.1 units, respectively, for clarity.

($T_c = 83$ K) was prepared by radio-frequency magnetron sputtering followed by a two-step post-annealing, first in air to promote epitaxial film growth, and second in argon to optimize the compositional stoichiometry¹⁸. A standard X-ray diffraction (XRD) measurement on our Tl2201 film shows only (00 l) reflection peaks (l up to 30 have been observed) indicating that the sample is phase-pure and has strong epitaxy. The ω -scan of the STO(200) reflection results in a double-peak rocking curve. Each peak has a full-width at half-maximum of $\sim 0.035^\circ$ with a splitting of $\sim 0.1^\circ$, suggesting that the precision in the c -axis alignment of the grains in the STO substrate is $\sim 0.1^\circ$. The rocking curve of the (00 $\bar{1}0$) reflection of the Tl2201 film has a full-width at half-maximum of 0.35° , indicating high-quality c -axis epitaxial film growth. ϕ -scan XRD measurements were made to assess the degree of in-plane alignment by monitoring the (105) reflection of the Tl2201 film and the (111) reflection of the STO substrate (Fig. 2). All of these XRD data indicate that both the substrate and the film are made of two parts, rotated with respect to each other about the c -axis by 45° , as intended. The ϕ -scan data also suggests that there is an $\sim 1^\circ$ misorientation in each grain at the boundary line MOM' (Fig. 1). We show below that this has no effect on the pairing symmetry test.

For a definitive conclusion of the present pairing symmetry test, it is essential to establish that the crystal structure of the Tl2201 films used here is tetragonal. However, it is impossible to distinguish between the tetragonal and orthorhombic structures by XRD data alone. The XRD signatures of orthorhombicity, such as the splitting of (110) tetragonal reflections into (020) and (200) peaks, are not observable in the c -axis oriented films, which are characterized only by the (00 l) reflections. The tetragonality of our Tl2201 epitaxial films was previously demonstrated by the combined use of indirect evidence from Raman spectroscopy and XRD¹⁸. More recently, the tetragonal crystal structure and the absence of twinning in these films have been well established by bright field imaging transmission electron microscopy, selected area electron diffraction, and convergent beam electron diffraction¹⁹.

We used a scanning SQUID microscopy (SSM)²⁰ to test the pairing symmetry. Details of our tricrystal experiments have been reported elsewhere^{3,4,8,21,22}. The SSM used in this experiment mechanically scans a sample with respect to a high-spatial-resolution, low- T_c integrated SQUID magnetometer so that the pickup loop is primarily sensitive to the z -component of the sample magnetic field, with a spatial resolution set by the size of the pickup loop. The SQUID magnetometer has an 8.2- μ m diamond-shaped pickup loop with 0.8- μ m linewidths and well shielded leads. Calibration of the magnetic field intensities in the tricrystal experiment geometry has been performed in a number of different ways which agree with each other to within $\sim 10\%$ (ref. 21).

Figure 3 shows a three-dimensional rendering of an SSM image of the Tl2201 blanket film deposited on a (100) SrTiO₃ substrate with the geometry of Fig. 1. There is only one magnetic vortex trapped in the $170 \times 230 \mu\text{m}$ field of view, at the bicrystal meeting point. Figure 4 shows cross-sections through the wedge point of the SSM image of Fig. 3 along the directions OA (curve a) and OB (curve b) as defined in Fig. 1. The observed field distribution was modelled as described in ref. 22; the surface fields of a Josephson vortex were propagated to the height of the pickup loop using Fourier transform techniques, and then integrated over the known pickup loop geometry. The close agreement between modelling and experiment for both wings of the vortex shows that this is indeed a $h/4e$ half-flux quantum and that the flux distribution is very symmetric, as designed. There is only the half-flux quantum trapped in a relatively large area, suggesting that this is spontaneously generated flux, as expected for the ground state of a superconducting loop with an odd number of sign changes in the pair tunnelling current. The magnetic flux trapped at the wedge tip is evenly and symmetrically distributed, with no extra flux spreading along the OM or OM'

directions, showing that the grain boundaries OM and OM' are strongly coupled: the sample acts as a bicrystal as intended.

The order parameter should transform in accordance with the symmetry operations of the relevant crystal point group. For a tetragonal system with point group C_{4v} such as Tl2201, s - and d -wave pair states correspond to two distinctly different irreducible representations A_{1g} and B_{1g} respectively. An admixture of s and d is not allowed. Therefore the observation of the half-integer flux quantum effect in this geometry represents a definitive, model-independent pair tunnelling evidence for pure $d_{x^2-y^2}$ pairing symmetry. Although the present experiment cannot distinguish between the $d_{x^2-y^2}$ and d_{xy} states, d_{xy} symmetry has been ruled out by photoemission^{23,24}. Our results can also unambiguously rule out symmetry-independent mechanisms, such as spin-flip tunnelling at the grain boundary, for the half-flux quantum effect. Such a mechanism would not have a sign reversal of I_s for the symmetry operation about the line MOM', and the half-integer flux quantum effect should not be observed. It should be pointed out that a mixed $s + d$ pair state is possible¹² if it is characterized by two bulk superconducting phase transitions. Such a double transition in the tetragonal system has not been observed. By varying the angle between the grain boundary OA and/or OB and the vertical line (Fig. 1) in the design of the wedge, one could determine the degree of $s + d$ admixture in an orthorhombic copper oxide such as YBCO by using the presence or absence of the half-integer flux quantum effect. $\square$

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Increased Australian wheat yield due to recent climate trends

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The possibility that future climate change may affect agriculture has attracted considerable attention^{1,2}. As a step towards evaluating such influences, the effect of climate trends over the past few decades³ needs to be assessed. Here I estimate the contribution of climate trends in Australia^{4,5} to the substantial increase in Australian wheat yields since 1952. Non-climatic influences—such as new cultivars and changes in crop management practices—are removed by detrending the wheat yield and climate variables and using the residuals to calculate quantitative relationships between variations in climate and yield. Climate trends appear to be responsible for 30–50% of the observed increase in wheat yields, with increases in minimum temperatures being the dominant influence. This approach should be applicable in other regions for which sufficient data exist.

The average yield of wheat, Australia's major crop, has increased by ~0.5 tonnes per hectare (0.5 t ha^{-1} ; ~45% of the average early 1950s yield of $\sim 1.1 \text{ t ha}^{-1}$) since 1952 (Fig. 1). One example of a climate variation that may influence wheat yield is frost frequency. The frequency of severe frosts has decreased in eastern Australia in recent decades⁶. Frosts inflict significant damage on wheat⁷, so that wheat crops in northeastern Australia are managed to avoid frost at flowering, where possible. The fewer frosts in recent years therefore may have contributed to the increased yield. Changes in rainfall and soil moisture would also affect yield, whereas an increase in mean daily temperature would decrease yield⁸. Here annual mean maximum and minimum temperatures (averaged across Australia) and annual average Australian rainfall totals have been related to Australian wheat yield. The climate data come from two new sets of high-quality data^{4,5}. The data have been carefully checked and adjusted, where necessary, for site changes and observational problems. Sites were chosen to ensure that urbanization did not contaminate the data. Mean annual maximum temperatures, averaged across Australia, increased by 0.58°C from 1952 to 1992. Mean annual minimum temperatures increased by 1.02°C , whereas mean annual rainfall only increased by 39 mm over the same period.

Although there are *prima facie* grounds for expecting that the observed climate trends should have affected Australian wheat yield, estimation of the actual effect requires removal of the possibly confounding effects of non-climatic factors. The approach adopted here to was to remove the effects of the trends in the yield and climate time-series by calculating first differences of the variables (that is, year-to-year changes) and then calculating the relationships between these first differences. The relationships can then be used to estimate what effect a trend in the climate variables should have had on yield. This approach avoids the confounding influence of long-term variations such as changes in crop management. Such factors will appear as 'noise' around the relationships between the year-to-year changes in yield and climate variables.

The correlation coefficient of the first differences of wheat yield with the first differences of rainfall was 0.57, with maximum temperature -0.56 , and with minimum temperature 0.12. The correlation with the first differences of the diurnal temperature range (maximum minus minimum) was -0.75 . The correlation with minimum temperature is not significant at 5%; the other correlations are significant at the 5% level or better. The tendency

for decreases in yield to be associated with increases in diurnal temperature range (and *vice versa*) is clear (Fig. 1), as is the 1952–92 trend (of -0.44°C) in diurnal range. The relationship between year-to-year variations is clearer in Fig. 2 which shows a scatter plot of the changes in wheat yield and diurnal range from year to year.

The linear regression between changes in yield (δW ; t ha^{-1}) and change in diurnal temperature range δD ; $^\circ\text{C}$) is shown in Fig. 2. It was calculated by forcing the intercept to pass through the origin (to avoid trend effects), and is given by $\delta W = -0.52 \delta D$. This equation was used to estimate the effect of the 1952–92 trend in diurnal temperature range on yield, with the assumption that a long-term change in diurnal range should affect crop yield to the same extent as would a change of this magnitude from one year to the next. The 1952–92 trend in diurnal range should have led to an increase in yield of 0.23 t ha^{-1} , under this assumption: that is, nearly half of the observed yield increase seems to be due to the decrease in the diurnal temperature range.

The above analysis does not use rainfall, nor does it separately include the effects of maximum and minimum temperatures. Multiple linear regression, with the year-to-year changes of wheat yield as the dependent variable, and year-to-year changes of rainfall, maximum and minimum temperatures as independent variables, has been used to estimate the separate effects of the three climate variables. The multiple correlation coefficient with the change in crop yield was 0.76. The multiple regression (again with intercept forced to zero, to remove a trend effect) was:

$$\delta W = -0.0004 \delta R + 0.5043 \delta T_{\min} - 0.6090 \delta T_{\max}$$

where δR is the change in rainfall (mm), $\delta T_{\min}$ is the change in minimum temperature ($^\circ\text{C}$) and $\delta T_{\max}$ is the change in maximum temperature ($^\circ\text{C}$). Substituting the 1952–92 trends in the three climate variables in this equation gives an estimate of a yield increase of 0.15 t ha^{-1} due to the climate trends, that is, ~30% of the observed increase in yield. The separate effects of each climate variable on the change in yield also can be estimated from the multiple regression. The very small trend in rainfall produced little change in yield. The increase in minimum temperature resulted in a large increase in yield (presumably reflecting less damage from frosts) which was partly offset by a decrease due to the increased maximum temperature.

The trend in minimum temperature has a larger effect on the yield than either rainfall or maximum temperature, even though the correlation between yield and minimum temperature was weaker

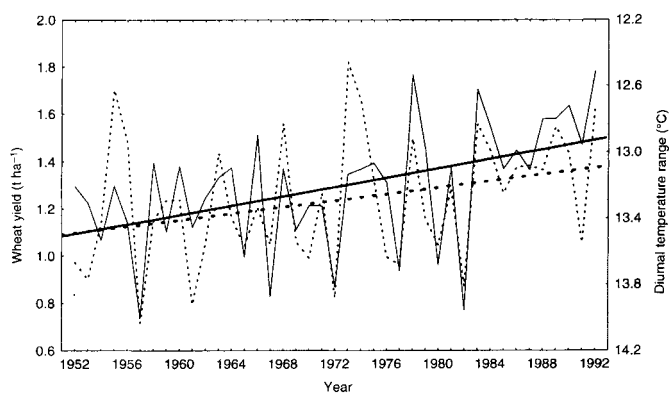


Figure 1 Plot of temporal variation in Australian average wheat yield (solid curve) and Australian annual average diurnal temperature range (dashed curve). The scale for the diurnal temperature range has been reversed, to facilitate comparison between the time series. The thick straight lines are the linear trends of the two variables (solid line, wheat yield; dashed line, temperature range).

than the correlations with the other two climate variables. This larger effect is partly due to the larger trend in minimum temperatures. More significant, however, is the fact that the three climate variables are closely correlated with each other. Year-to-year changes in maximum temperature have a correlation of -0.55 with first differences of rainfall and 0.57 with first differences in minimum temperature. If the partial correlations are calculated between first differences of the yield and each of the climate variables, thereby removing the influence of other two climate variables, then the important underlying influence of minimum temperatures is revealed. The partial correlations of first differences of yield with the first differences of rainfall, minimum temperature, and maximum temperature are -0.10 , 0.57 and -0.58 , respectively. The strong underlying relationship between minimum temperature and yield is reflected in the coefficients in the multiple regression of yield against the climate variables. Changes in rainfall have only a weak effect on yield, once the cross-correlations between rainfall and temperature are taken into account through multiple regression or partial correlation.

Other methods for removing the trends in the yield and climate variables, other than using year-to-year changes, could be used before calculating the relationships between climate and yield. For instance, the calculation using the regression of yield versus diurnal temperature range was repeated, this time using residuals from simple exponential smoothing, instead of year-to-year changes. This approach gave an estimate of the effect of the 1952–92 trend in diurnal temperature range on wheat yield of 0.17 t ha^{-1} , intermediate between the two trends estimated above using first differences.

The estimates provided here suggest that Australian wheat yield should have increased, as a result of climate trends, by about 10–20% since the middle of the century, if other factors had been held constant. This increase is about 30–50% of the total increase in wheat yield. This estimated increase should include the 'direct' effects of climate on Australian agriculture (for example, fewer frosts causing less damage to wheat). The analysis should not have been confounded by the gradual increase in atmospheric CO_2 levels and the associated fertilization effect^{1,2}. The direct CO_2 effect on yield would be included in that part of the increase not identified here as related to the climate variations and trend. But to check that CO_2 variations were not confounding the analysis, year-to-year changes in CO_2 atmospheric concentration at Mauna Loa⁹ (for the period 1958–92) were included, with the climate variables, as

predictors in the multiple regression with wheat yield. The correlations between year-to-year changes of CO_2 and yield were small (compared with the strong correlations of yield and the climate variables) and not statistically significant. The inclusion of CO_2 concentrations in the regression calculations resulted in only insignificant changes to the estimates of the yield changes due to the climate trends.

The technique used here involves the removal of trends in dependent variable ('impact variable') and independent variable, calculation of regression of detrended variables, and the use of this regression to estimate effect of trend on dependent variable. It could be used to estimate the effect of climate change in other areas, on other crop yields, or on other impact variables such as malaria cases. It is not an alternative to more complex methods used for estimating the future impacts of predicted climate change¹⁰, because there is no guarantee that the relationships between impact variables and climate variables will remain constant in the future. It does, however, provide a simple approach to estimating the impact of recent climate trends, with the assumption that a climate variation of a certain magnitude will lead to the same impact irrespective of whether the climate variation occurred from one year to the next or over several decades.

This approach was also applied to estimate the effects of climate trends on the total value of Australian crops (converted to constant 1995/96 dollars). Crop value variations are caused by, *inter alia*, changes in crop management, and national and global economic variations. Climate variations also affect total crop value. For instance, the correlation between the first differences of the diurnal temperature range and total Australian crop value is -0.70 . Repeating the analysis used on the wheat yields suggests that the total value of Australian crops should have increased by about 15–30%, since mid-century, due to the trends in climate variables. The effects of other factors, including global economic activity as well as local crop management, appear as the noise in the regression between the first differences of Australian crop value and of Australian climate variables. Only factors with first differences which are closely related to the first differences of Australian climate variables would confound this analysis.

One possible confounding factor which may not be accounted for here is the reaction of farmers to growing conditions. If farmers respond to good years, by increasing fertilizer use for instance, this may exaggerate the impact of the climate variables on yield. However, farmers becoming aware of a trend to better conditions would also presumably increase their inputs. So, some increased yield would arise from the increased inputs in better conditions. □

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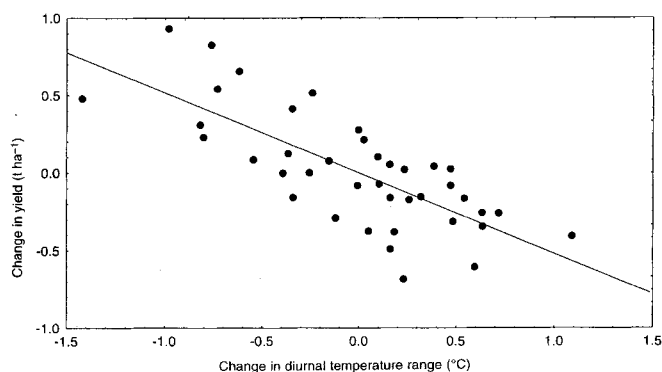


Figure 2 Scatter diagram of year-to-year changes in Australian wheat yield versus year-to-year changes in Australian diurnal temperature range. The line indicates the linear regression between the two variables. Data from 1952/3 to 1991/2.

A new tetragonal silicate mineral occurring as inclusions in lower-mantle diamonds

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Aluminium is one of a small group of elements—Si, Mg, Fe, Al, Ca and O—that form the bulk of the Earth's mantle. In the upper mantle, Al is largely contained in minerals with a garnet structure. But the mineral transformations associated with the breakdown of garnet structures in the uppermost lower mantle have been a matter of uncertainty^{1–9}. Here we report the discovery of a new aluminous mineral, present as inclusions in diamonds of lower-mantle origin found at São Luiz, Brazil¹⁰. This phase has a stoichiometric garnet composition similar to pyrope–almandine but has a distinct tetragonal structure (space group *I42d*), and is here referred to by the acronym TAPP (tetragonal almandine–pyrope phase). Although TAPP has not been recognized in experimental studies, we suggest that it is nevertheless a primary rather than retrogressive phase and has a limited stability field in relatively aluminous bulk compositions in the uppermost lower mantle. TAPP, like garnet, would lead to relatively low densities for basic rocks compared with peridotites (assumed to be the dominant mantle rocks), and we suggest therefore that it may play an important role in determining density differences and the dynamics of segregation between ultrabasic and basic compositions in the mantle^{7,11,12}.

Diamonds from São Luiz have been reported¹⁰ to contain inclusions of silicate minerals with the compositions of (Mg,Fe)SiO₃ and CaSiO₃, together with inclusions of the oxide (Mg,Fe)O. These compositions indicate an assemblage of the minerals (Mg,Fe)-perovskite, Ca-perovskite and ferropericlasite, which has been predicted¹³ and shown by experiments^{6,9,14} to be stable for expected mantle bulk compositions only below depths of the upper-to-lower mantle transition at ~660 km. Such assemblages also yield values of seismic wave velocities consistent with those observed for the lower mantle^{14,15}. In addition to the occurrence of the association (Mg,Fe)SiO₃, CaSiO₃ and (Mg,Fe)O from São Luiz, Mössbauer measurements on Fe oxidation states in the ferropericlasite inclusions support a lower-mantle origin^{16,17}, while X-ray studies have also shown extremely high confinement pressures on ferropericlasite inclusions within São Luiz diamonds¹⁸.

The new phase, TAPP, has been found to occur in eight diamonds from São Luiz and was first reported¹⁰ as a phase of lower-mantle paragenesis because of its association with (Mg,Fe)SiO₃ and (Mg,Fe)O inclusions. Its chemical composition, as determined by electron microprobe analysis, is closely similar to that of minerals with garnet structure and the mineral was initially described as a garnet¹⁰. Of the TAPP inclusions identified by chemical analysis, two occurred alone in diamond and five coexisted with separate inclusions of ferropericlasite (including one case where TAPP was also in direct contact with a (Mg,Fe)SiO₃ inclusion). In another case, lacking ferropericlasite, TAPP was in contact with a Mg, Al, Na silicate. The inclusions of TAPP composition were recovered from

diamonds free of obvious surface-to-inclusion fractures, and in two instances windows were polished on the diamond to confirm the absence of fractures from the inclusion to the diamond surface. Thus, the inclusions are considered to be syngenetic with their diamond hosts and not of extraneous origin. The inclusions were extracted from the diamond by careful crushing of the diamond host.

The TAPP crystals are 30–100 µm in diameter and of apple green colour. They exhibit either a distinct cubo-octahedral morphology, or in one case an elongate and tabular form. Cubo-octahedral morphologies often arise in mineral inclusions in diamonds because the diamond imposes its own morphology on the inclusions during a period of mutual growth¹⁹.

Representative analyses showing the chemical range in composition of TAPP grains are shown in Table 1. All eight crystals have very similar compositions with the exception of BZ207A, which is Fe–Ti rich but still shows a similar stoichiometry. The TAPP compositions are very similar to pyrope–almandine garnet compositions, although the ratio of Mg/(Mg + Fe) cations (~0.93) would be unusually high for naturally occurring garnets. The TAPP compositions notably show normal garnet Si : Al ratios without evidence of a majorite type of substitution that affects some mantle garnets. Compared with other aspects of mantle garnet compositions, the new phase also shows unusually low CaO in combination with low Cr₂O₃. Thus, despite having garnet stoichiometry, TAPP belongs to a different compositional field to mantle garnets. The Fe³⁺ content of TAPP is high as seen on the basis of both calculation from electron probe data and from Mössbauer analyses by McCammon *et al.*¹⁷, who obtained Fe³⁺/(Fe²⁺ + Fe³⁺) = 0.778 and 0.648 for samples BZ238A and BZ243A respectively. Finally, the results of ion probe analyses show very low concentrations ((0.01–1) × chondrite) of large-ion lithophile, rare-earth and high-field-strength elements²⁰ in the new phase.

Sample BZ244B was examined using a diffractometer (equipped with a fast area scanning television detector system, FAST TV) with Mo Kα radiation, and sufficient single-crystal data were recorded to achieve a full structural determination. Crystallographic data and methods are given in Table 2. The structure determination has shown the tetragonal nature of TAPP with space group *I42d*. It contains three independent oxygen sites in general positions, two distinct tetrahedral 'silicon' sites, both with two-fold symmetry, and three other cation sites; M1 has four-fold symmetry and a 'capped' tetrahedral environment, with four short and four long M1–O bonds, whilst M2 and M3 have two-fold symmetry, with octahedral coordination. The Si tetrahedra are not linked together, so that TAPP is an orthosilicate. On the basis of independent sites, it can be described by the formula: (M1)₁(M2)₂(M3)₂(Si1)₁(Si2)₂(O1)₄(O2)₄(O3)₄ which can be simplified to (M1)(M2)₂(M3)₂Si₃O₁₂.

Using the chemical data for major elements (Table 1), coupled with known crystallochemical behaviour, and the results of many least-squares refinement trials and bond-valence calculations, cation assignments were made as follows. (1) The two types of Si sites were modelled to achieve full occupancy by mixing in ~4.5% Al. Ferric iron was considered as a possibility for balancing the Si deficiency in the Si sites but was not consistent with the observed bond lengths. (2) Six crystallochemically controlled model configurations were considered for the siting of the remaining Al³⁺, the Mg²⁺ and the transition-metal elements. The best refinement placed Al³⁺, Cr³⁺ and Mn²⁺ in the smaller octahedral site M2; Fe²⁺ into M3; Mg²⁺ distributed between predominantly the octahedral M3 site and the 'capped' tetrahedral M1 site; and Fe³⁺ solely in the 'capped' tetrahedral site M1. This assignment is supported by values for isomer shift and quadrupole splitting for Fe²⁺ and Fe³⁺ calculated on the basis of the Mössbauer analyses¹⁷.

The full site allocations are given in Table 2, but the broad result of this refinement using principal cations gives a formula of

(Mg, Fe³⁺)[Al, Cr, Mn]₂[Mg, Fe²⁺]₂Si₃O₁₂ where (), [] and { } indicate M1, M2 and M3 sites respectively.

The chosen model leaves small deficiencies at all M sites, and so some minor switching of cations could be possible. The refinement results, however, can be judged to be quite satisfactory according to all normal criteria (such as *R* values and thermal parameters). In view of such good fit and of the constraints we have been able to

apply (in terms of analytical results and cation charge sums), our proposed model must be close to the actual structure. Some of the bond lengths are relatively long, but it is quite possible that expansion of the crystal structure has occurred following the release of the specimen from its diamond enclosure, and any extraneous bond elongation could be expected to be taken up by TAPP under its natural conditions of high pressure and temperature. Observations

Table 1 Electron microprobe analyses for three representative grains of TAPP

Sample	206B	207A	244B		206B	207A	244B
Analysis	5	4	2				
Wt%				Cations			
SiO ₂	42.43	39.56	42.12	Si	2.92	2.81	2.91
TiO ₂	0.01	4.20	0.06	Ti	0.00	0.22	0.00
Al ₂ O ₃	23.48	20.16	23.83	Al	1.90	1.69	1.94
Cr ₂ O ₃	2.22	1.39	2.80	Cr	0.12	0.07	0.15
FeO	4.64	9.41	4.60	Fe ²⁺	0.08	0.16	0.08
				Fe ³⁺	0.19	0.40	0.19
MnO	0.47	0.25	0.96	Mn	0.03	0.02	0.06
NiO	0.07	0.03	0.01	Ni	0.00	0.00	0.00
NaO	0.15	0.03	0.09	Na	0.02	0.00	0.01
MgO	26.66	24.85	25.63	Mg	2.73	2.63	2.64
CaO	0.12	0.03	0.09	Ca	0.01	0.00	0.01
K ₂ O		0.00	0.00	K	0.00	0.00	0.00
Total	100.24	99.91	100.20	Total	8.00	8.00	8.00

Major-element compositions were determined on a Cameca Camebax electron microprobe at the University of Edinburgh, Dept of Geology and Geophysics. The probe was operated at 20 kV with a beam current of 20 nA. Fe³⁺ values were calculated on the basis of Fe³⁺/(Fe³⁺ + Fe²⁺) = 0.714, averaged from Mössbauer data.

Table 2 Crystallographic data for TAPP

Positions and thermal parameters*						
Atom†	Site	x	y	z		
M(1)	4a	0	0	0		
M(2)	8d	0.2600(4)	0.2500	0.1250		
M(3)	8c	0	0.5000	-0.0227(1)		
Si(1)	4b	0.5000	0.5000	0		
Si(2)	8d	-0.1508(4)	0.2500	0.1250		
O(1)	16e	0.0186(7)	0.2794(7)	0.0573(2)		
O(2)	16e	-0.2621(8)	0.0374(6)	0.1011(2)		
O(3)	16e	0.4370(7)	0.2954(7)	0.0460(2)		
	U ₁₁	U ₂₂	U ₃₃	U ₁₂	U ₁₃	U ₂₃
M(1)	3(1)	3(1)	10(1)	0	0	0
M(2)	9(1)	7(1)	4(2)	0	0	0(1)
M(3)	5(2)	5(2)	6(1)	-2(2)	0	0
Si(1)	8(2)	8(2)	6(2)	0	0	0
Si(2)	8(2)	7(2)	10(2)	0	0	1(1)
O(1)	7(3)	8(3)	9(2)	-3(3)	-2(2)	2(2)
O(2)	13(3)	10(2)	10(2)	6(3)	-2(2)	4(2)
O(3)	8(3)	16(4)	6(2)	6(2)	1(2)	-2(2)
Interatomic distances (Å) and angles (°)						
M(1)-O(1)	2.109(5)	(×4)	M(1)...O(2)	2.521(5)	(×4)	
M(2)-O(1)	2.004(5)	(×2)	M(2)-O(2)	1.925(4)	(×2)	
M(2)-O(3)	1.866(5)	(×2)	M(3)-O(1)	2.052(5)	(×2)	
M(3)-O(2)	2.124(5)	(×2)	M(3)-O(3)	2.014(5)	(×2)	
Si(1)-O(3)	1.630(5)	(×4)	Si(2)-O(1)	1.664(5)	(×2)	
Si(2)-O(2)	1.625(5)	(×2)				
O-M(1)-O	104.3(1), 120.4(2)		O...M(1)...O	86.48(1), 122.05(2)		
O-M(2)-O ^{cis}	103.7(3), 90.8(2), 88.7(2), 89.9(2), 87.9(2), 92.8(2), 76.5(3)		O-M(2)-O ^{trans}	179.2(4), 166.3(2)		
O-M(3)-O ^{cis}	93.7(2), 103.6(2), 89.4(3), 79.6(2), 84.1(2), 87.7(2), 95.4(3)		O-M(3)-O ^{trans}	155.7(3), 175.8(2)		
O-Si(1)-O	118.3(3), 105.2(1)		O-Si(2)-O	126.8(4), 101.7(2), 113.2(2), 96.4(3)		

Tetragonal; space group $I4_2d$ (no. 122); $a = 6.526(4)$ Å, $c = 18.182(9)$ Å. Crystal dimensions, $0.06 \times 0.04 \times 0.04$ mm; formula weight 417.36; calculated density, 3.580 Mg m^{-3} , Delft Instruments Fast, 293(2) K, Mo $K\alpha$ radiation ($\lambda = 0.71069$ Å), graphite monochromator; $\mu = 1.854 \text{ mm}^{-1}$; 1,581 reflections (322 independent, $I > 4\sigma$); data/parameters = 6/8; $R = 0.039$, $R_w = 0.071$. The structure was determined via direct methods (SHELX-86[†]) and refined by the least-squares program SHELX-93[‡].

* All atoms refined with anisotropic thermal parameters. The anisotropic displacement factor exponent takes the form: $-2\pi^2[h^2a^{*2}U_{11} + \dots + 2hka^*b^*U_{12}]$, where a^* and b^* are reciprocal lattice parameters.

† M(1) = Mg²⁺ 0.19025, Fe³⁺ 0.0465; M(2) = Al³⁺ 0.44000, Cr³⁺ 0.0380, Mn²⁺ 0.01425; M(3) = Mg²⁺ 0.46975, Fe²⁺ 0.02025; Si⁴⁺ 0.4787, Al³⁺ 0.02125.

of expansion on release from diamond have been made for ferro-periclase inclusions¹⁸.

The evidence that São Luiz inclusions involving the combination of (Mg,Fe)O, (Mg,Fe)SiO₃ and CaSiO₃ phases are of lower-mantle origin is compelling, but this still leaves open the important question as to whether TAPP is a primary phase in this lower-mantle association or is a retrogressive relict formed under decompression from an origin lower-mantle mineral.

Lower-mantle minerals and high-pressure phases are characteristically of a high density, containing cation sites with large coordination numbers⁹. The calculated density of TAPP at atmospheric pressure is 3.580 g cm⁻³ and marginally less than that of a garnet-structured phase of similar composition with 3.634 g cm⁻³. Coordination in the new phase—three four-fold, four six-fold and one distorted eight-fold ('capped' tetrahedron) sites per formula unit—suggests a relatively open structure compared to normal garnet which has three distorted eight-fold, two six-fold and three four-fold sites. But in the absence of any values of bulk modulus or compressibility for TAPP, it is uncertain how its density will compare with garnet at depth in the Earth. If TAPP has a relatively high compressibility, then its density might become greater than that of garnet at high pressure. We also note that the garnet structure is extremely widespread across the range of upper-mantle and crustal pressure-temperature conditions and compositions. Thus if retrogression of a separate very high pressure phase of pyrope-almandine composition occurred, it seems most likely that it would have given rise to the garnet rather than the TAPP structure. The virtual absence of Ca in TAPP is a reflection of it not having a suitable site for Ca or any other large ion; this is because the 'capped' tetrahedral M1 site has a significantly smaller volume than the [8] site in garnet. The absence of such a site in a mineral from the lower-mantle association is appropriate given the stability of a specific Ca-bearing phase in the form of Ca-perovskite.

If we accept TAPP to represent a primary lower-mantle phase distinct from garnet, we must consider its possible phase relations in more detail and why no evidence of it has been found in experimental studies so far. High-pressure experiments show that, in the lower part of the upper mantle, Al is essentially located in a majoritic garnet (relatively low in Al compared with normal garnet), and with passage from upper mantle to lower mantle and the stabilization of (Mg,Fe)- and Ca-perovskites, this garnet becomes less abundant. With increasing depth in the uppermost region of the lower mantle, much recent experimental work^{1-3,5,6} shows evidence of increasing substitution of Al into (Mg,Fe)-perovskite. As a consequence, bulk rock compositions which are low in Al, such as common mantle peridotites, are expected to gradually become garnet-free in the uppermost 60 km (from 660 to 720 km depth) of the lower mantle^{1,6}.

For bulk compositions containing more Al, such as basic rock compositions close to pyrope-almandine garnet itself, the situation is less clear-cut. Several experimental studies^{4,7,9,21-23} have found evidence of an aluminous phase distinct from garnet, but these new phases have not been fully characterized and we note that none of them have X-ray diffraction or chemical compositional features that correspond well to TAPP. In general the broadest consensus of evidence^{1-3,5} supports increasing substitution of Al into (Mg,Fe)-perovskite with depth, and it seems likely that normal (Mg,Fe)-garnet compositions become essentially represented by perovskite somewhere in the depth range 800–1,350 km (refs 1–3). At these and greater depths, strongly aluminous compositions may contain corundum^{1,2,5}.

The reasons why TAPP has not so far been identified in experimental work possibly include the following. (1) Its stability field may only span a small range of pressures (and temperatures). (2) Over the span of pressure-temperature conditions concerned, TAPP may only occur in a restricted range of bulk compositions, which may not coincide with those of common peridotitic bulk

compositions and might depend on other particular features (for example, the Fe:Mg or ferrous:ferric ratios). (3) Many of the experimental runs which have been carried out on obviously appropriate bulk compositions, such as those on the same compositions as pyrope-almandine garnet, have been done using an actual garnet as starting material¹⁻⁵, and under the short run times of these difficult experiments, metastable persistence of the garnet may have hindered the nucleation and growth of TAPP. (4) The characterization of phases in very fine-grained run products can be very difficult.

With such considerations in mind, and the strong experimental evidence in favour of extensive Al substitution into (Mg,Fe)-perovskite with depth in the lower mantle, it seems most likely that TAPP stability occurs in the uppermost part of the lower mantle. If its stability field overlaps with the high-pressure part of the stability field for garnet with majorite substitution, or with the field in which aluminous (Mg,Fe)-perovskite forms the aluminous phase in peridotite, TAPP may well be restricted to more aluminous basic compositions. Under such circumstances TAPP, like garnet⁷, may be expected to lead to relatively low densities for basic rocks compared with peridotites. In terms of mantle dynamics^{7,11,12,14}, therefore, TAPP may play an important part in the development of density differences between subducted basic compositions and peridotitic compositions, which will affect their relative segregation and circulation in the mantle. □

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Evidence for a clade of nematodes, arthropods and other moulting animals

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The arthropods constitute the most diverse animal group, but, despite their rich fossil record and a century of study, their phylogenetic relationships remain unclear¹. Taxa previously proposed to be sister groups to the arthropods include Annelida, Onychophora, Tardigrada and others, but hypotheses of phylogenetic relationships have been conflicting^{2,3}. For example, onychophorans, like arthropods, moult periodically, have an arthropod arrangement of haemocoel^{1,4}, and have been related to arthropods in morphological and mitochondrial DNA sequence analyses^{4,5}. Like annelids, they possess segmental nephridia and muscles that are a combination of smooth and obliquely striated fibres⁶. Our phylogenetic analysis of 18S ribosomal DNA sequences indicates a close relationship between arthropods, nematodes and all other moulting phyla. The results suggest that ecdysis (moulting) arose once and support the idea of a new clade, Ecdysozoa, containing moulting animals: arthropods, tardigrades, onychophorans, nematodes, nematomorphs, kinorhynchans and priapulids. No support is found for a clade of segmented animals, the Articulata, uniting annelids with arthropods. The hypothesis that nematodes are related to arthropods has important implications for developmental genetic studies using as model systems the nematode *Caenorhabditis elegans* and the arthropod *Drosophila melanogaster*, which are generally held to be phylogenetically distant from each other.

We have analysed relationships of arthropods to other taxa by sequencing complete 18S rDNAs from representative taxa, aligning them with existing 18S sequences from other metazoan taxa, and analysing them by using standard phylogenetic techniques⁷. This study confirms the suspected relationships between arthropods and other taxa, such as tardigrades and onychophorans. But by careful consideration of rates of evolution, we find the surprising result that nematodes are also closely related to arthropods.

An outstanding problem with the molecular phylogeny of nematodes is that their 18S sequences evolve too rapidly to be useful for phylogenetic reconstruction. Previously published sequences of nematodes have a substitution rate 2–3 times greater than those of most other Metazoa. Hence special efforts were made to include only the slowest evolving sequences from representative taxa, because errors due to unequal rate effects and alignment artefacts are compounded by including rapidly evolving sequences⁸. To obtain three slowly evolving nematode sequences, 10–20 nematode 18S genes were sequenced (J.R.G., unpublished results). Marked differences are observed, depending upon whether rapidly or slowly evolving sequences are present (Fig. 1). When both rapidly and slowly evolving nematode sequences (bold type) are included, all nematodes branch from the base of the bilateral animals (Fig. 1a), whereas, when only the slowest nematode sequence is included

(Fig. 1b), the nematode branches high within the protostomes as the sister taxon of the arthropods. Furthermore, analysis of the slowly evolving protein-synthesis elongation factor EF-1 α also place nematodes within the protostomes (J.R.G., A.M.A.A. and J.A.L., unpublished results), suggesting that other evolutionary processes⁹ are not responsible. These results are consistent with unequal rates artefactually placing rapidly evolving, long-branched nematode sequences adjacent to the long branch that joins the outgroup to the tree. Molecular sequence analysis, using the available fast-evolving 18S rRNA nematode sequences or faster evolving molecules, has demonstrated a similarly deep placement¹⁰, whereas some morphological studies have predicted a placement similar to that found using only the slowly evolving nematode sequence¹¹.

To exclude rapidly evolving taxa, all complete 18S rDNA sequences relevant to this study were systematically surveyed. An alignment of about 50 of the most useful complete sequences was constructed and the distances from each taxon to the last common ancestor of protostomes was calculated using the paralinear/LogDet method^{12,13} (Table 1). Guided by these distances, the slowest evolving protostome and outgroup taxa were selected (shown in bold). These included the slowest evolving sequences from the following taxa: a cnidarian as an outgroup to triploblastic animals^{7,14}, a deuterostome as an outgroup to the protostome animals, a polychaete, an oligochaete, a brachiopod, a mollusc, a non-moulting aschelminth, representatives of the six phyla of non-arthropod moulting animals, and four major arthropod groups (a chelicerate, a crustacean, a myriapod and an insect).

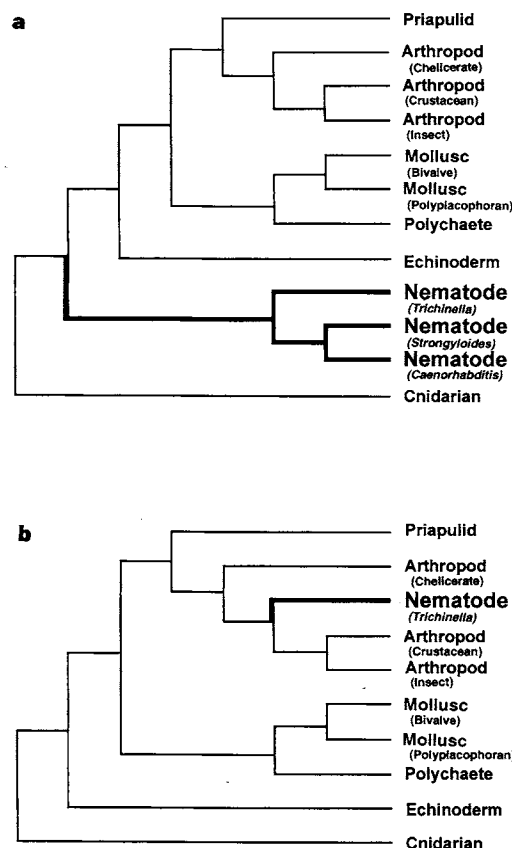


Figure 1 Phylogenetic analysis of 18S rDNA sequence data illustrating the effects of unequal rate biases on nematode placement. In **a**, both rapidly and slowly evolving nematode sequences (*Caenorhabditis* and *Strongyloides*, and *Trichinella*) are included in the analysis; the nematodes branch from the bottom of the tree, even before the deuterostome-protostome divergence. In **b**, only the slowly evolving *Trichinella* sequence is included and this nematode now branches from within the protostome clade, as the sister taxon to the arthropods.

The majority-rule consensus tree derived from phylogenetic reconstructions is shown in Fig. 2. Four reconstruction methods were used, including parilinear (LogDet) distances^{12,13}, maximum parsimony⁹, Kimura two-parameter distances²⁸, and Jukes–Cantor distances²⁸. Parilinear (LogDet) distances^{12,13} were emphasized because of their generality (most distance methods are special cases of parilinear distances). As preliminary calculations indicated an excess of constant sites (see Methods), all distance methods were corrected for site-to-site variation. Bootstrap values for these four methods, respectively, are shown adjacent to the interior nodes.

In all of the reconstructions, the protostome taxa are clustered into two monophyletic groups. One clade, containing all the moulting animals (kinorhynch, priapulid, nematomorph, onychophoran, nematode, tardigrade, crustacean, insect, myriapod and chelicerate) is present in 95, 78, 85 and 79% of the trees derived through parilinear distances, maximum parsimony, Kimura two-parameter and Jukes–Cantor distances, respectively. The other protostome clade, containing the articulate brachiopod, mollusc, oligochaete, polychaete and rotifer, is present in 98, 80, 99 and 100%

of the bootstrap replicates. A monophyletic protostome clade is also supported in 97, 83, 96 and 95% of the bootstrap replicates. Interpreted using the empirical results of Hillis and Bull¹⁵ as a guideline, these data provide significant support ($P \leq 0.05$) for a clade of arthropod-related moulting animals within the protostomes. This conclusion is further supported by topology-dependent cladistic permutation tail probability tests confirming the significance of the arthropod-related clade ($P \leq 0.01$).

We initially found that flatworm sequences, like rapidly evolving nematode sequences, branched below the base of the bilateral animals. Hence multiple flatworm taxa were sequenced in order to obtain slowly evolving 18S sequences. In experiments similar to those shown in Fig. 1 (with flatworm sequences substituted for nematode sequences), flatworms were shown to branch artefactually deep. Given the importance of the phylogenetic position of the platyhelminthes to theories of the evolution of bilateral animals^{16,17}, a tree containing slowly evolving lophotrochozoal taxa and the most slowly evolving flatworm, *Stenostomum*, was reconstructed (Fig. 3). Bootstrap support for the clade consisting of the flatworm and other lophotrochozoans is high (91, 88, 83 and 80%, for parilinear distances, maximum parsimony, Kimura two-parameter and Jukes–Cantor distances, respectively), consistent with the placement of the flatworms within the Lophotrochozoa¹⁸.

Divisions within the protostomes have long been a major point of contention among zoologists. Conventional wisdom supports the

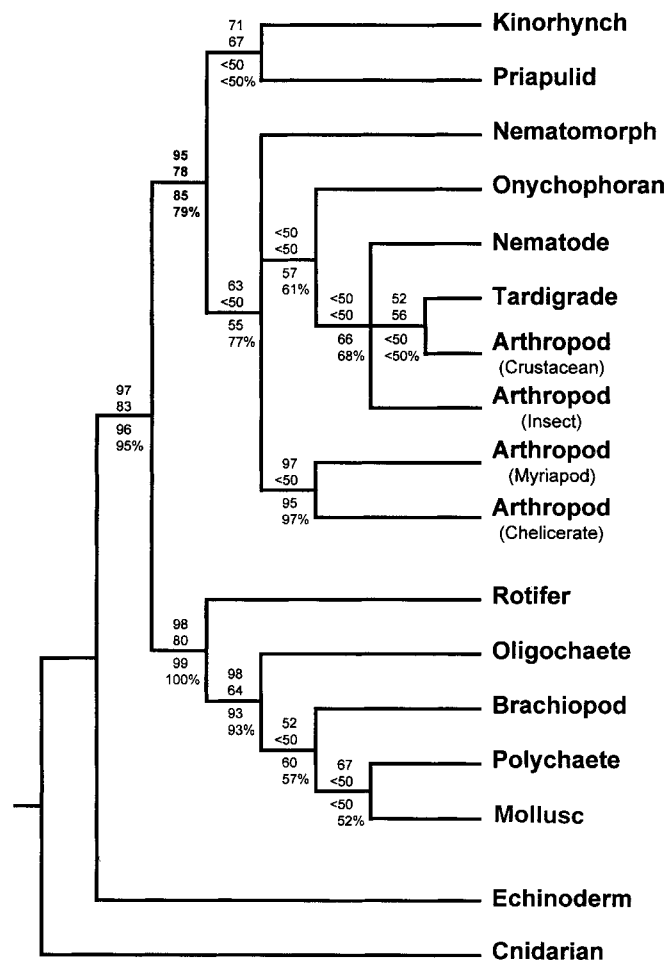


Figure 2 Phylogenetic analysis of 18S rDNA sequence data to determine relationships among the moulting metazoans. The moulting animals are present as the top ten taxa, the Lophotrochozoa are shown in the middle, and outgroups are shown at the bottom. The topology shown here is a majority-rule consensus combining the results from four individual majority-rule consensus trees derived using the following methods: parilinear/LogDet distances, maximum parsimony, Kimura two-parameter distances and Jukes–Cantor distances. All distance methods are corrected for site-to-site variation. The numbers next to the central branches represent the percentage of bootstrap replicates supporting the clades for these methods, respectively (from top to bottom).

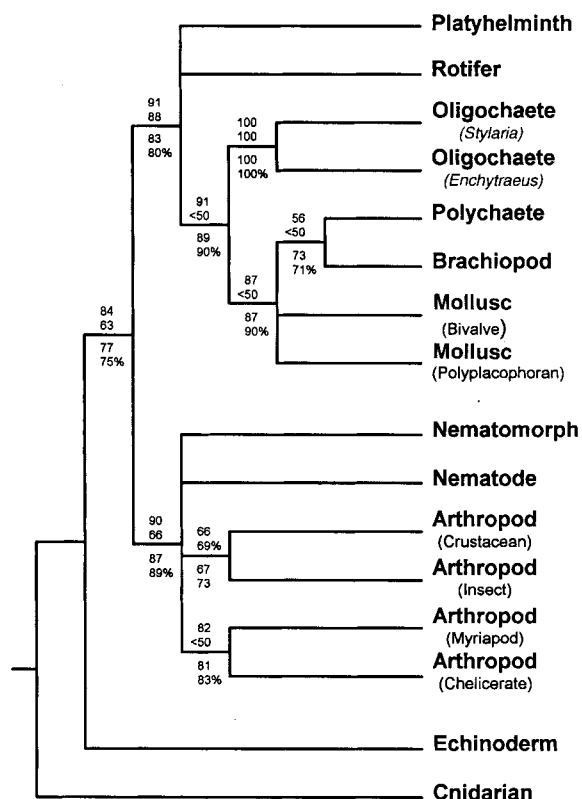


Figure 3 Phylogenetic analysis of 18S rDNA sequence data to illustrate relationships of the flatworm to other protostome animals. The Lophotrochozoa are present as the top eight taxa, the Ecdysozoa are shown in the middle, and outgroups are shown at the bottom. The topology shown here is a majority-rule consensus combining the results from four individual majority-rule consensus trees derived using the following methods: parilinear/LogDet distances, maximum parsimony, Kimura two-parameter distances and Jukes–Cantor distances. All distance methods are corrected for site-to-site variation. The numbers next to the central branches represent the percentage of bootstrap replicates supporting the clades for these methods, respectively (from top to bottom).

existence of a clade, the Articulata, that includes the segmented animals, chiefly the arthropods and the annelids. This concept has a long tradition, but has been called into question by analysis of morphological and palaeontological data^{3,19} and of 18S rRNA sequence data^{7,20,21}. Eernisse *et al.* characterized two clades within the protostomes, the arthropods and the Eutrochozoa (annelids, molluscs and other protostomes developing from a trochophore larva) with morphological data³. A number of studies using 18S data^{7,14,20,21,30} identified two clades within the protostomes, the arthropods and the coelomate protostomes of Field *et al.*, now called Lophotrochozoa⁷. The lophotrochozoans include the annelids, molluscs, rotifers, phoronids, brachiopods, bryozoans, platyhelminthes and related phyla. Our data indicate that the sister clade to the lophotrochozoans contains the remaining protostomes, which all develop by moulting. Segmentation does not seem to be a synapomorphy uniting annelids and arthropods. Our analyses, which

include four aschelminths (pseudocoelomates), do not support aschelminth monophyly, in agreement with molecular studies^{2,10,30}. Our studies are consistent with the clade cephalorhyncha¹⁶.

Our interpretation of these results is shown in Fig. 4. The most obvious feature of this phylogeny is that it separates the protostomes into two groups, an arthropod-related clade exclusively composed of animals that moult, and a lophotrochozoal clade exclusively containing non-moulting animals. All members of the arthropod-related clade undergo ecdysis²². In addition, all members lack locomotory cilia, although other groups (for example, chaetognaths and acanthocephalans) also lack them¹⁶. Given the observed tree topology and these common structural features, this raises the possibility that ecdysis and the cellular modifications associated with it may have been derived only once within this clade.

Because these 18S rDNA analyses support the hypothesis that all moulting animals (arthropods, tardigrades, onychophorans, nematodes, nematomorphs, kinorhynchs and priapulids) share a common ancestor to the exclusion of deuterostomes and the lophotrochozoans, we have chosen the node-based name²³ Ecdysozoa. This group is defined as these taxa plus their last common ancestor and all of its descendants. The name reflects the property that all members of this group, and only members of this group, undergo ecdysis during at least part of their life cycles.

It was unexpected to find nematodes contained within the Ecdysozoa because in previous molecular studies they diverged deep in the protostome tree, even before the deuterostome-protostome bifurcation¹⁰. Boore *et al.*²⁴, in their pioneering study using mitochondrial gene order, assumed that nematodes were an outgroup to the protostomes. We realized the results of previous molecular studies could be unequal rate artefacts caused by the extremely rapid nucleotide-substitution rates found in previously published rhabditid nematode sequences, and therefore sequenced numerous nematode species to identify slowly evolving representatives. Unequal rate effects are well documented in theory¹⁵

Table 1 Substitution rates of 18S rDNA sequences

Phylum	Genus	Substitutions per site
Lophotrochozoa		
Chaetognatha	<i>Sagitta</i>	0.143 ± 0.111
Sipuncula	<i>Phascolosoma</i>	0.079 ± 0.007
Pogonophora	<i>Siboglinum</i>	0.070 ± 0.008
Platyhelminthes	<i>Bdelloura</i>	0.147 ± 0.012
	<i>Fasciolopsis</i>	0.083 ± 0.009
	Stenostomum	0.063 ± 0.063
Nemertea	<i>Lineus</i>	0.061 ± 0.007
Echiura	<i>Ochetostoma</i>	0.058 ± 0.007
Vestimentifera	<i>Ridgeia</i>	0.055 ± 0.007
Mollusca	<i>Lymnaea</i>	0.060 ± 0.006
	Placopecten (bivalve)	0.042 ± 0.007
	Acanthopleura (polyplacophoran)	0.040 ± 0.006
Aschelminthes:		
Acanthocephala	<i>Moniliformis</i>	0.111 ± 0.009
Gastrotricha	<i>Lepidodermella</i>	0.070 ± 0.007
Rotifera	Brachionus	0.058 ± 0.007
Lophophorates:		
Phoronida	<i>Phoronis</i>	0.053 ± 0.007
Ectoprocta	<i>Plumatella</i>	0.049 ± 0.006
Brachiopoda	<i>Glottidia</i>	0.044 ± 0.006
	Terebratalia	0.044 ± 0.006
Annelida	<i>Eisenia</i>	0.057 ± 0.007
	<i>Lanice</i>	0.056 ± 0.006
	Enchytreus (oligochaete)	0.052 ± 0.006
	Stylaria (oligochaete)	0.042 ± 0.006
	Glycera (polychaete)	0.033 ± 0.005
Arthropods and relatives		
Nematoda	<i>Strongyloides</i>	0.192 ± 0.014
	<i>Caenorhabditis</i>	0.187 ± 0.013
	<i>Trichuris</i>	0.141 ± 0.012
	Trichinella	0.110 ± 0.010
Onychophora	Euperipatoides	0.090 ± 0.009
Tardigrada	<i>Milnesium</i>	0.079 ± 0.008
	Macrobatus	0.079 ± 0.009
Kinorhyncha	Pycnophyes	0.075 ± 0.007
Nematomorpha	Gordius	0.068 ± 0.007
Arthropoda	<i>Artemia</i>	0.068 ± 0.007
	Panulirus (crustacean)	0.065 ± 0.008
	<i>Drosophila</i>	0.121 ± 0.011
	<i>Crossodonthina</i>	0.056 ± 0.007
	Tenebrio (insect)	0.048 ± 0.006
	Scolopendra (myriapod)	0.043 ± 0.006
	<i>Androctonus</i>	0.046 ± 0.006
	Eurypelma (chelicerate)	0.038 ± 0.005
Priapula	Priapulid	0.040 ± 0.005
Outgroups		
Chordata	<i>Lampetra</i>	0.065 ± 0.007
	<i>Branchiostoma</i>	0.059 ± 0.006
Echinodermata	<i>Strongylocentrotus</i>	0.043 ± 0.006
	Antedon	0.040 ± 0.005
Ctenophora	<i>Mnemiopsis</i>	0.130 ± 0.111
Cnidaria	<i>Anemonia</i>	0.101 ± 0.009
	Tripedalia	0.100 ± 0.009

Distances are calculated by paraligner/LogDet distances and the $\pm$ s.d. estimated from bootstrap replicates. The number of substitutions per position from the last common ancestor of protostomes was calculated with respect to three slowly evolving reference taxa. Distances to protostome taxa were calculated using *Tripedalia* and *Antedon* as outgroup taxa and either *Glycera* or *Priapulid*, depending upon which ingroup taxon was being examined. Distances to outgroup taxa were calculated using *Glycera*, *Priapulid* and *Acanthopleura* as reference taxa.

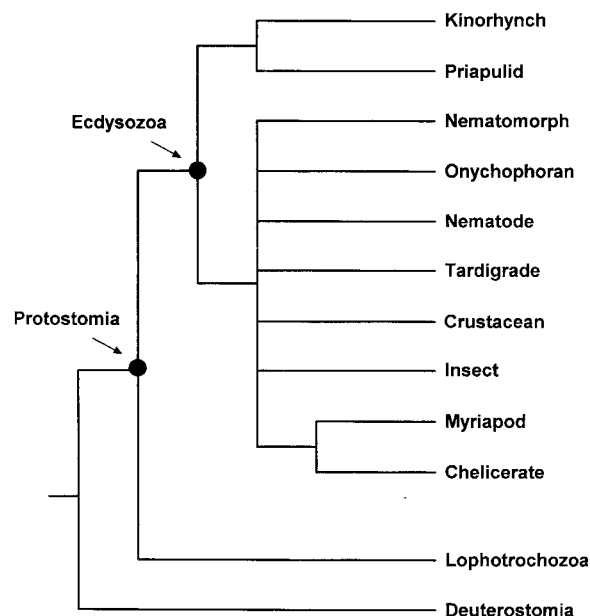


Figure 4 As inferred from 18S rDNA, the Protostomia consists of two major groups. The Lophotrochozoa includes the lophophorates, molluscs, annelids, rotifers and other groups⁷. The Ecdysozoa includes the arthropods, tardigrades, onychophorans, nematomorphs, nematodes, kinorhynchs, priapulids and probably the loriciferans. (So far, no living specimens and fewer than 200 preserved loriciferans (which moult) have been collected. Morphological evidence, however, suggests a close relationship to kinorhynchs and priapulids^{16,29}.) The common ancestors of these clades are indicated.

but are usually ignored. Morphological studies also support the inclusion of nematodes with many ecdysozoans, although not with arthropods^{11,16}. One thoughtful analysis groups nematodes, nematomorphs, priapulids, kinorhynch and loricifera (but not arthropods, onychophorans and tardigrades) using the synapomorphies, "loss of locomotory cilia, cuticle moulted, introvert with spines, teeth or scalds"¹⁶. (These first two synapomorphies also serve to unite the ecdysozoa.) Another recent cladistic analysis of morphological characters supports a clade of moulting animals excluding the priapulids³, although nematomorphs were not included in that analysis.

Given the tremendous interest in the nematode *Caenorhabditis elegans* and the arthropod *Drosophila melanogaster* as model systems, the hypothesis that both are closely related has important implications for developmental and genomic studies. For example, it has been assumed that developmental mechanisms common to *Caenorhabditis* and to *Drosophila* originated before the protostome–deuterostome divergence and hence should also be found in *Homo sapiens*. Our results imply that mechanisms found in both nematodes and fruitflies will not necessarily be found in humans.

The inclusion of the priapulids within an arthropod-containing clade was not anticipated because most morphological studies had not indicated a close priapulid, arthropod phylogenetic relationship^{2,3}. Both arthropods and priapulids are numerically prominent members of the Burgess shale faunas²⁵, indicating the early success (and successful preservation) of ecdysozoans in the Cambrian radiation.

These studies provide evidence that the nematodes are not primitive metazoans but are protostomes related to arthropods. They also support a monophyletic protostome clade. Considering the greatly differing morphologies, embryological features and life histories of the moulting animals, it was initially surprising that the ribosomal RNA tree should group them together. However, given that all moulting taxa sampled are in this clade, and given the significant anatomical modifications associated with moulting, such as the lack of locomotory cilia, ecdysis appears to be a defining synapomorphy for this group, although additional molecular data from other molecules are necessary to test further or confirm the monophyly of the moulting animals. □

Methods

DNA isolation. Total genomic DNA was isolated by standard techniques and amplified by the polymerase chain reaction (PCR). PCR fragments or complete sequences were then cloned into a plasmid vector before sequencing. Replicates of the PCR amplification were sequenced in both directions. A list of the PCR and sequencing oligonucleotides and PCR reaction conditions is available from J.A.L. or J.R.G. (gary@chuma.cas.usf.edu).

Sequences. The following sequences are available in GenBank: *Brachionus plicatilis* (Rotifer; accession number, U49911), *Enchytraeus* sp. (Oligochaete; accession number, U95948), *Euperipatoides leukartii* (Onychophoran; accession number, U49910), *Gordius* sp. (Nematomorph; accession number, U51005), *Macrobrius* sp. (Tardigrade; accession number, U49912), *Milnesium tardigradum* (Tardigrade; accession number, U49909), *Stenostomum* sp. (Platyhelminth; accession number, U95947), *Stylaria* sp. (Oligochaete; accession number, U95946), and *Trichinella spiralis* (Nematode, accession number, U60231).

Sequence alignments. An alignment of 49 complete sequences was constructed using the star alignment procedure to reduce biases⁸, with the slowly evolving *Glycera americana* sequence used as the reference, and then proofread by hand. Pairwise alignments of nucleotide sequences were performed with the ALIGN program, using a break penalty of 6; nucleotide identities, transversions and transitions were scored as +3, +1 and 0, respectively, based on preliminary experiments with EF-1 α and 18S rDNA. Regions were excluded from the analysis if extreme length variation existed among sequences, or if many of the sequences contained gaps that could be easily moved with little or no change in alignment score. The alignments are available from J.A.L.

Phylogenetic reconstruction. The 17-taxon phylogenetic trees shown in Figs 2 and 3 were obtained using PAUP version 3.1.1 for maximum parsimony analyses and Bootstrappers gambit²⁶ for distance analyses. For both methods, 200 bootstrap trees were calculated to determine the 50% majority-rule consensus tree; each search was initiated with 100 replicates of random taxon addition, and positions with gaps were excluded. For parsimony, the following heuristic search options were used: starting trees were obtained by stepwise addition (starting seed was 1) with one tree held at each step; and tree-bisection-reconnection branch-swapping was performed with the MULPARS, but not the steepest descent, option. For paralogous/LogDet, Kimura two-parameter and Jukes–Cantor distances, four-point metrics were used to assess quartet values; the quartet consistency value²⁶ (53.46%) was selected to ensure that the probability of finding the best solution was >99.9%. A cnidarian and an echinoderm were used as the outgroups, except in Fig. 3 where the two slowest echinoderms were used for parsimony to further reduce unequal rate effects. As site-to-site variation was judged to be significant, distances were corrected for this artefact by estimating nine site categories from the data, calculating distances from the eight non-categories, and estimating trees from the sums of the distances¹².

Site-to-site variation. Site-to-site variation was considered significant when estimated using a diagnostic statistical test for the number of constant sites²⁷. Maximum-likelihood trees were calculated using the DNAML program (version 3.4) in PHYLIP. Parameters necessary for the test were calculated for a variety of substitution models using both single and double rate categories determined by the hidden Markov model²⁸. An excess of observed constant sites (overpredicted sites) was found for all models, indicating that even two-site categories could not fully explain the data. (Using empirical base frequencies and a transition/transversion ratio of 2.0, the best single-site model (rate ratio, 2:1; probability of each rate, 0.5, 0.5) predicted 787 $\pm$ 39 site versus 1,081 observed sites, and the best two-category model (rate model, 10:1; probability of each rate, 0.8, 0.2) predicted 963 $\pm$ 38 sites versus 1,081 observed sites. All choices of parameters reconstructed trees with monophyletic ecdysozoal and lophotrochozoal clades, although long computation times prevented bootstrap analysis.)

Bootstrap interpretations. Based on empirical studies of bootstrap analyses, they represent highly conservative estimates of phylogenetic accuracy. Typically for maximum parsimony, bootstrap proportions of $\geq 70\%$ correspond to a probability of $\geq 95\%$ that the respective clade is a historical lineage. For Gambit, the probabilities are slightly less conservative.

T-PTP test. The topology-dependent cladistic permutation tail probability (T-PTP) test determines whether the difference in length between the shortest tree supporting the monophyly of this clade and the shortest tree not supporting monophyly (5 steps difference) is significantly different from the difference in length expected from randomized data. If the difference in length between the monophyly and non-monophyly trees is outside 95% of the distribution based on randomized data, it can be concluded that the data significantly support monophyly of the clade. We used 200 randomized data sets that were analysed by maximum parsimony.

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An ancestral mitochondrial DNA resembling a eubacterial genome in miniature

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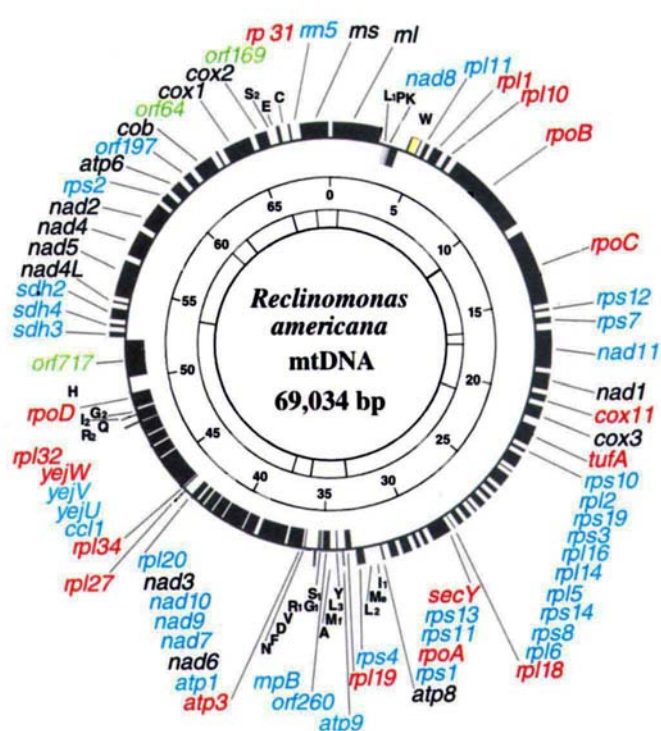
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Mitochondria, organelles specialized in energy conservation reactions in eukaryotic cells, have evolved from eubacteria-like endosymbionts^{1–3} whose closest known relatives are the rickettsial group of α -proteobacteria^{4,5}. Because characterized mitochondrial genomes vary markedly in structure³, it has been impossible to infer from them the initial form of the proto-mitochondrial genome. This would require the identification of minimally derived mitochondrial DNAs that better reflect the ancestral state. Here we describe such a primitive mitochondrial genome, in the freshwater protozoan *Reclinomonas americana*⁶. This protist displays ultrastructural characteristics that ally it with the retortamonads^{7,8}, a protozoan group that lacks mitochondria^{8,9}. *R. americana* mtDNA (69,034 base pairs) contains the largest collection of genes (97) so far identified in any mtDNA, including genes for 5S ribosomal RNA, the RNA component of RNase P, and at least 18 proteins not previously known to be encoded in mitochondria. Most surprising are four genes specifying a multisubunit, eubacterial-type RNA polymerase. Features of gene content together with eubacterial characteristics of genome



ultrastructural characteristics with the amitochondriate retortamonads (refs 7, 8 and C.J.O'K., M. A. Farmer and T. A. Nerad, manuscript in preparation), a group that is thought to have diverged from the main eukaryotic line before the acquisition of mitochondria⁹. Thus the jakobid flagellates may be ancestral, mitochondria-containing protists that represent an early offshoot of the eukaryotic lineage.

The AT-rich (74%) sequence of *R. americana* mtDNA (69,034 base pairs (bp)) assembles into a unique unicircular map, densely packed with genes that are distributed on both strands (Fig. 1). Intergenic spacer sequences make up only 8% of the mitochondrial genome, and only a single (group II) intron has been identified, within the *trnW* gene. There are a total of 92 genes to which a function can be assigned, including all of the 44 protein-coding genes previously found in one or more sequenced mtDNAs (Table 1), as well as 26 tRNA genes and 3 rRNA genes. Among the latter is a 5S rRNA gene¹⁰ that until now had been found only in the mtDNAs of land plants¹¹ and the unicellular chlorophyte alga, *Prototheca wickerhamii*¹². Also present are two unidentified open reading frames (ORFs) that are conserved in plant and some other protist mtDNAs, as well as three unique ORFs of >60 codons (see Fig. 1).

The 26 predicted tRNA species all have conventional secondary structures, with very few deviations from the canonical structure. They have the potential to read all codons used in *R. americana* mitochondrial protein-coding genes with the exception of ACN (threonine). The lone tryptophan tRNA gene (*trnW*) has a CCA

anticodon, which is consistent with the use of the standard genetic code in *R. americana* mitochondria.

In addition to protein-coding and RNA structural genes already known from other mitochondrial genomes, *R. americana* mtDNA contains 18 genes of assignable function (identified by searches against public-domain sequence databases) that have not previously been reported in mtDNA (Table 1). These new mtDNA-encoded genes include one (*atp3*) that encodes an additional component of the electron transport chain, nine new mitoribosomal proteins (all large subunit), and a gene (*yeyW*) that specifies a fourth component of a cytochrome *c*₁ biosynthesis pathway. Genes of a type never before found in mtDNA include ones encoding a translation factor (*tufA*), a secretory pathway protein (*secY*), a putative cytochrome oxidase assembly protein (*cox11*), and four components of a eubacterial-type ($\alpha_3\beta\beta'\sigma$) RNA polymerase (*rpoA-D*).

The presence of eubacteria-like *rpo* genes in *R. americana* mtDNA is especially intriguing, considering that the only mitochondrial RNA polymerase so far identified is a nucleus-encoded, single-polypeptide enzyme homologous to bacteriophage T3 and T7 RNA polymerases^{13,14}. Given the evidence supporting a eubacterial, endosymbiotic origin of the mitochondrion¹⁻⁵, it is not obvious how a single-subunit, T3/T7-like RNA polymerase came to be present in this organelle, and why it is used in mitochondrial transcription instead of a multisubunit, eubacteria-like enzyme, homologous genes for which are encoded in all characterized

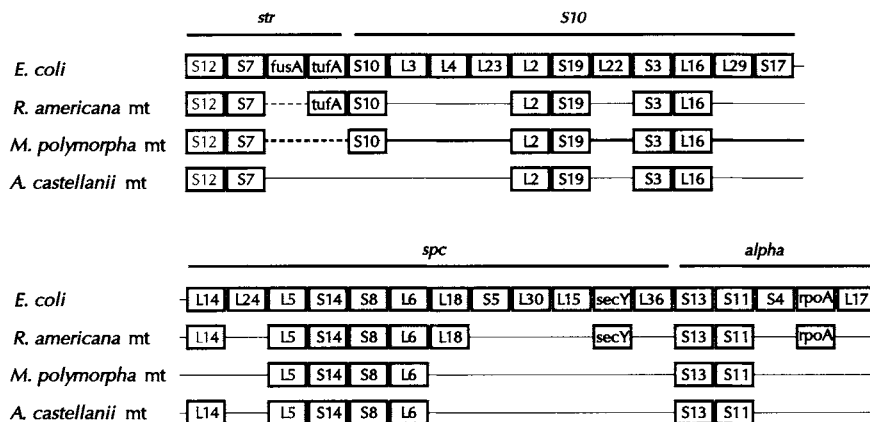


Figure 2 Conservation of ribosomal protein gene organization in the mitochondrial (mt) genomes of *Reclinomonas americana*, *Marchantia polymorpha* (liverwort) and *Acanthamoeba castellanii*, compared to the contiguous strepto-

mycin (*str*), S10, spectinomycin (*spc*) and alpha operons of *E. coli*. Solid lines connect adjacent genes, dashed lines indicate the presence of additional genes that are not shown.

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AAACTATAAGAAATAGTTATTTAAAAA AAAGGA AAAAAAATG..... tufA
TATGTGGGATTTCTTTGTGTGCTTAGAG AAAGGAATGTATATTTTATG... nad1
TTAATGTTTTTAATATTTTGTATAGT AAAGGA AATAAGATG..... nad11
TTAATTATTTAATATTTTATTATTTT AAAGGATTCTTATATG..... rps7
ATTCAAAGAAAATATTATAAATAAATATGGAATTTATTTTATG..... rpoB
ATAGAAGTGTGTTGATTTTAAATATAA AAAGGTTGGTATAATATG..... nad4L
GTTTCAAACCTTATTTTAAAGTAAATG AAAGGATATTTATATG..... cox2
TATAATTATAAATTCATAGAAAATAAAA AAAGATAATCGCTTATG..... cox1
TATTGTATTTATAAATAAATAAAAAAAC AAAGGA AATTCAAATG..... cox3
*****
3' UUUCU CAAGUAGGUCGA...SSU rRNA

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Figure 3 Putative Shine-Dalgarno motifs (red) immediately upstream of inferred translation initiation codons (ATG, shown in blue) in representative *R. americana* protein-coding sequences. Potential base pairing between inferred Shine-Dalgarno sequences and the 3'-terminal region the *R. americana* mitochondrial

SSU rRNA (green) is indicated by asterisks. The motif 5'-AAAGGA-3' or one differing from it by a single nucleotide precedes at least 50 of the protein-coding sequences in *R. americana* mtDNA.

chloroplast genomes^{15,16}. Although additional work will have to be carried out to verify that the *R. americana* *rpo* genes are functional, their sequences do not suggest that they are inactive pseudogenes.

Regardless of whether the *R. americana* mitochondrial *rpo* genes are actually functional, the simplest explanation for their presence is that they derive directly from the α -proteobacterial ancestor of mitochondria. If this is so, their absence from the mtDNA of most other eukaryotes implies that they were lost from the proto-mitochondrial genome at an early stage in mitochondrial evolution and were functionally replaced by a nuclear gene encoding a single polypeptide, T3/T7-like enzyme.

These observations prompt several questions. What was the evolutionary source of the T3/T7-like RNA polymerase now used as the mitochondrial RNA polymerase in many, if not most, eukaryotes? Is a multisubunit, eubacteria-like RNA polymerase used instead of (or in addition to) a T3/T7-like enzyme for mitochondrial transcription in some organisms, notably the jakobid flagellates? If so, might genes for such a eubacteria-like enzyme have moved into the nucleus in some cases? We presume that at some point in the evolution of mitochondria, recruitment of a nucleus-encoded, single polypeptide, T3/T7-like RNA polymerase allowed subsequent loss of the multisubunit, mtDNA-encoded activity, with eventual elimination of the corresponding genes from the mitochondrial genome. In this regard, it would be of interest to know whether T3/T7-like RNA polymerase sequences exist in *Reclinomonas* nuclear DNA (nDNA). A recent polymerase chain reaction (PCR) survey¹⁷ provided evidence of such sequences in a number of unicellular eukaryotes, but not in *R. americana* or several other protists.

The *R. americana* mitochondrial genome encodes another typical eubacterial protein, a homologue of SecY, a component of the Sec protein-sorting machinery found in all eubacteria and plastids examined to date¹⁸. The presence of *secY* in *R. americana* mtDNA suggests that a Sec-based sorting pathway may act in the transport of proteins in *Reclinomonas* mitochondria. However, an intensive search of the completely sequenced yeast nuclear genome has failed to reveal any nDNA-encoded Sec homologues in *Saccharomyces cerevisiae*¹⁹. This situation may represent another instance of early loss from the proto-mitochondrial genome of genetic information for a eubacterial pathway, and its replacement by a new, nDNA-encoded one.

Vestiges of a prokaryotic operon organization are clearly evident in several gene clusters in *R. americana* mtDNA. These include the linkage groups *rpoB-rpoC*, *nad3-nad10-nad9-nad7*, *nad4L-nad5-nad4-nad2*, *sdh3-sdh4-sdh2*, and *yejW-yejV-yejU-ccl1* (= *yejR*). The most extensive bacteria-like linkage is found in a cluster of ribosomal protein genes that corresponds to the contiguous *str*, *S10*, *spc* and α operons of *Escherichia coli* (Fig. 2). By comparison with

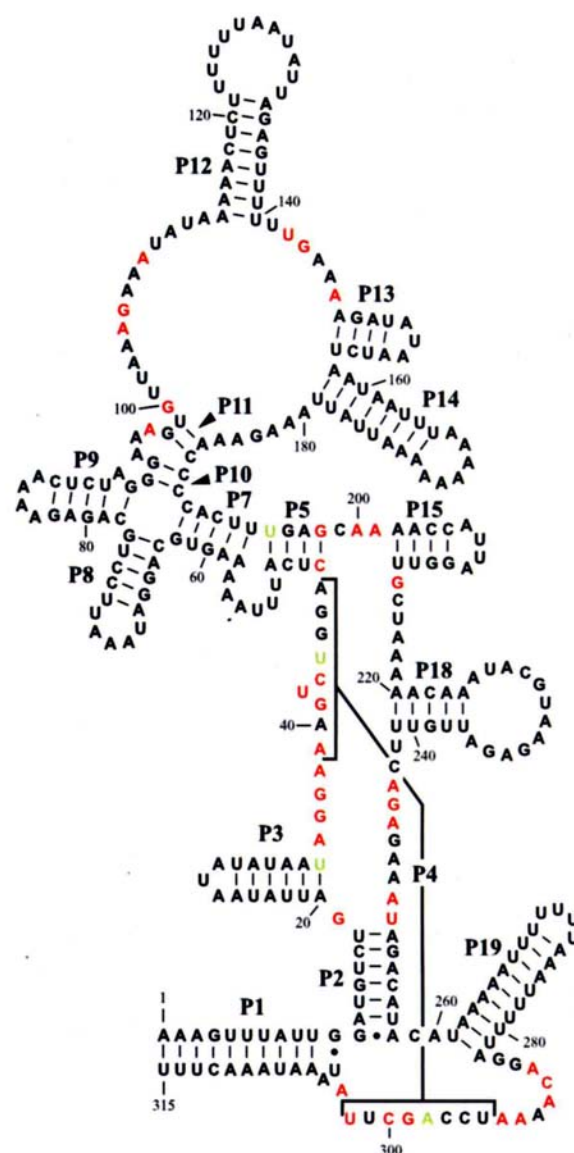


Figure 4 Secondary structure of *R. americana* RNase P RNA. The model is based on the *E. coli* secondary structure, and helical regions are numbered accordingly²⁸. Residues in red match the bacterial consensus model²⁸, whereas those in green do not. A long-range tertiary interaction (P4) is supported by compensating base changes at two positions, relative to the *E. coli* model. (Figure courtesy of J. R. Brown, Indiana University).

Table 1 Protein-coding genes of identified function in *Reclinomonas americana* mitochondrial DNA

Electron transport and ATP synthesis	
Complex I	<i>nad</i> 1, 2, 3, 4, 4L, 5, 6, 7, 8, 9, 10, 11
Complex II	<i>sdh</i> 2, 3, 4
Complex III	<i>cob</i>
Complex IV	<i>cox</i> 1, 2, 3
Complex V	<i>atp</i> 1, 6, 8, 9, 3
Translation	
Small subunit ribosomal proteins	<i>rps</i> 1, 2, 3, 4, 7, 8, 10, 11, 12, 13, 14, 19
Large subunit ribosomal proteins	<i>rpl</i> 2, 5, 6, 11, 14, 16 1, 10, 18, 19, 20, 27, 31, 32, 34
Elongation factor	<i>tufA</i>
Transcription	
Core RNA polymerase	<i>rpo A, B, C</i>
Sigma-like factor	<i>rpoD</i>
Protein import/maturation	
Cytochrome oxidase assembly protein	<i>cox11</i>
SecY-type transporter	<i>secY</i>
ABC transporter	<i>yej R</i> (=cc11), <i>U</i> , <i>V</i> , <i>W</i>

Genes in bold and underlined are unique to *R. americana* mtDNA; the remainder have previously been found in mtDNA in other eukaryotes. An additional five protein-coding genes of unknown function are present in *R. americana* mtDNA (see Fig. 1).

clusters containing the same genes in the mitochondrial genomes of *Marchantia polymorpha*²⁰ and *Acanthamoeba castellanii*²¹, it can be seen that the *Reclinomonas* cluster retains genes that have been deleted from one or both of the other two mtDNAs. These retained genes include *rpl18* as well as the non-ribosomal protein genes *tufA*, *secY* and *rpoA*. But it is striking that the three mtDNAs share specific deletions compared to the corresponding *E. coli* operons, including *rpl3-rpl4-rpl23* (between *rps10* and *rpl2*), *rpl22* (between *rps19* and *rps3*), *rpl29-rps17* (between *rpl16* and *rpl14*), *rpl24* (between *rpl14* and *rpl5*) and *rps5-rpl30-rpl15* (downstream of *rpl6*). Other specifically mitochondrial linkage groups are shared among these three eukaryotes; these clusters include *nad4-nad2-rps2* (in both *R. americana* and *A. castellanii* mtDNAs) as well as *sdh4-(sdh2)-nad4L* (ref. 22). Taken together, these comparisons suggest that various mitochondrion-specific features of gene organization had already been established in the mitochondrial genome of the most recent common ancestor shared by *Reclinomonas* and other eukaryotes. This conclusion strongly reinforces the concept of a single origin of the mitochondrial genome^{5,23}, arguing against the idea that *R. americana* might have acquired its relatively conserved mitochondrial genome through a different, more recent endosymbiotic event.

As a further indication of eubacterial character, we note that a Shine-Dalgarno-type interaction²⁴ is theoretically possible in the mitochondrial translation system of *Reclinomonas*, whereas this is not the case in other eukaryotes. In the *Reclinomonas* mitochondrial small subunit (SSU) rRNA, a pyrimidine-rich sequence occurs at the same position as the anti-Shine-Dalgarno sequence in *E. coli* 16S rRNA (the inferred 3'-end of the *Reclinomonas* SSU rRNA is 5'-CUCCUUU_{OH}, compared to 5'-CUCCUUA_{OH} in *E. coli* 16S rRNA). A complementary purine-rich sequence (often 5'-AAAGGA-3') is located between 2 and 12 nucleotides upstream of the inferred initiation codon of most protein-coding genes in *Reclinomonas* mtDNA (Fig. 3).

Finally, a gene encoding an RNase P RNA is present in *R. americana* mtDNA. Such a gene has previously been identified only in the mtDNA of *S. cerevisiae* and a few other fungi²⁵, including most recently *Aspergillus nidulans*²⁶. In contrast to the very AU-rich fungal mitochondrial RNase P RNAs²⁷, the inferred *R. americana* homologue is strikingly eubacterial, displaying almost all of the evolutionarily conserved primary sequence and secondary structural motifs of a phylogenetic-minimum bacterial consensus RNase P RNA²⁸ (Fig. 4). At an estimated size of 311 nucleotides (nt), the *R. americana* RNase P RNA is only slightly larger than its smallest known eubacterial homologue, that from *Mycoplasma fermentans* (276 nt)²⁸. Like the *Mycoplasma* RNase P RNA, the *Reclinomonas* one has an extra helix (P19) near the 3'-end and lacks helices P16 and P17. However, unlike the *Mycoplasma* homologue, the *Reclinomonas* RNase P RNA retains helices P12, P13 and P14 of the *E. coli* secondary structure (Fig. 4).

The 69-kbp *R. americana* mtDNA encodes a total of 67 protein-coding genes (including two unidentified but conserved ORFs and three unique ORFs), compared with 470 protein-coding regions in the 580-kbp genome of the eubacterium, *Mycoplasma genitalium*²⁹, and 1,743 protein genes in the 1.8-kbp *Haemophilus influenzae* genome³⁰. Thus protein-coding gene density is similar in the three genomes (1 gene per 1.0–1.2 kbp). Comparison of the *Mycoplasma* and *Haemophilus* genomes suggested that their different gene contents reflect "profound differences in physiology and metabolic capacity between these two organisms"²⁹, the differential reduction and tailoring of genetic information presumably being driven by the particular biological niches occupied by these two bacteria. In this context, the *Reclinomonas* mitochondrial genome may be viewed as an extreme example of eubacterial genome reduction, such that the only genes remaining are related to mitochondrial gene expression (transcription, RNA processing and translation) and biogenesis of the protein complexes required for electron transport

and coupled oxidative phosphorylation (including components implicated in mitochondrial protein transport and haem biosynthesis).

In summary, *R. americana* mtDNA provides a striking example of a minimally derived mitochondrial genome, one that offers new insights into gene content, organization and expression in the ancestral proto-mitochondrial genome. *R. americana* mtDNA contains the largest gene set uncovered to date, with many new genes, including some for new mitochondrial functions. Of particular note is the first documented presence of *rpo* and *sec* genes in mtDNA, a finding that has implications for our views about the origin and evolution of the mitochondrial transcriptional apparatus and protein import machinery. *R. americana* mtDNA displays more pronounced eubacterial features of gene organization (linkage) and gene expression (Shine-Dalgarno potential) than any other sequenced mtDNA, further testifying to the eubacterial ancestry of the mitochondrial genome. Continued exploration of mitochondrial genomes within the most ancestral unicellular eukaryotes can be expected to refine further our understanding of the nature of the most recent common ancestor of extant mitochondrial genomes and the divergent pathways mitochondrial genome evolution has since taken in different eukaryotic lines. □

Methods

The sequence of the *R. americana* mitochondrial genome has been determined under the auspices of the Organelle Genome Megasequencing Program (OGMP). Details of growth conditions for *R. americana* and isolation and cloning of its mtDNA will be presented in conjunction with a more detailed description and analysis of the complete sequence (G.B. *et al.*, unpublished results). DNA sequencing, data entry and sequence analysis were performed as described²¹.

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Behavioural stress facilitates the induction of long-term depression in the hippocampus

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The induction of activity-dependent persistent increases in synaptic efficacy, such as long-term potentiation (LTP), is inhibited by behavioural stress^{1,2}. The question arises whether stress also affects the ability to induce persistent decreases in synaptic efficacy, such as long-term depression (LTD)^{3–5}. We now report that the induction of stable homosynaptic LTD in the CA1 area of the hippocampus of awake adult rats is facilitated, rather than inhibited, by exposure to mild naturalistic stress. The same stress blocked the induction of LTP. The effects of such stress were short lasting: acclimatization to, or removal from, the conditions that facilitated LTD induction led to a rapid loss of the ability to elicit this form of plasticity. The time window in which LTD could be reliably elicited was prolonged by inducing anaesthesia immediately after the stress. These data reveal that even brief exposure to mild stress can produce a striking shift in the susceptibility to synaptic plasticity in the awake animal.

The conditions necessary to induce LTD in freely moving awake animals have proved elusive^{6–8}. As exposure to inescapable aversive stimuli has been reported to reduce high-frequency stimulation (HFS)-induced LTP in the hippocampus^{1,2}, we wondered whether stress would also affect the ability to elicit LTD. To investigate this, three experimental protocols that produce mild naturalistic stress were compared with three related but non-stressful protocols.

The first set of experiments compared the ability of low-frequency stimulation (LFS) to induce LTD in recording-acclimatized (non-stressed) and recording-naïve (stressed) rats. Animals that had been acclimatized extensively to the brightly lit recording box and recording procedure after electrode implantation showed no signs of behavioural stress and had low serum levels of corticosterone. In contrast, rats that had not been handled during the 2-week period after surgery showed behavioural signs of stress, including behavioural 'freezing' (remaining in a fixed immobile position), defecation and urination for up to 10 min on first being placed in the brightly lit unfamiliar recording box. They also had raised serum corticosterone (see Methods). Consistent with previous studies^{6–8} in acclimatized animals, LFS (900 pulses at 3 Hz) of the ipsilateral Schaffer collateral/commissural fibres in the stratum

radiatum of the CA1 region did not affect the amplitude of the field excitatory postsynaptic potential (EPSP) (Fig. 1a), although LTP was reliably induced by high-frequency stimulation (200 Hz; Fig. 1b). In contrast, reliable LTD was induced in stressed animals. Thus, when the LFS protocol was applied to unhandled, recording-naïve animals 40 min after they had been placed for the first time in the recording box, stable homosynaptic LTD was elicited (Fig. 1c; see also Fig. 3c). Consistent with previous reports on the effects of stress^{1,2}, LTP induction was blocked in recording-naïve rats (Fig. 1d). Significantly, the stress of transferring recording-naïve animals from the home cage to the new environment did not produce a baseline change in synaptic transmission at the time of recording that might have affected the ability to induce LTD (Fig. 1e).

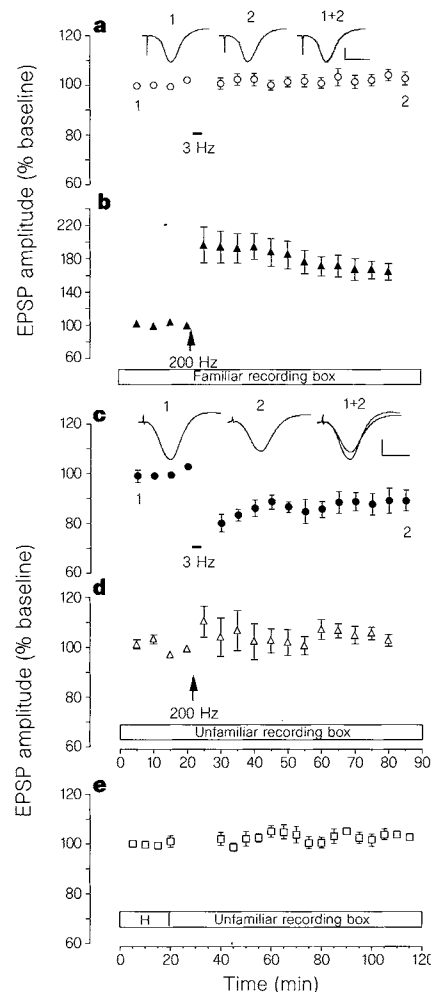


Figure 1 Novelty stress enables low-frequency stimulation (LFS) to induce LTD and prevents high-frequency stimulation (HFS) eliciting LTP in freely moving, recording-naïve rats. **a**, LFS (3 Hz; bar) failed to induce LTD of the field EPSP amplitude in rats that had been acclimatized to the recording box and procedure ($n = 10$; $101.8 \pm 2.4\%$ of baseline 30 min after LFS). Inset: field potentials (average of 10 consecutive sweeps) from one experiment at the times indicated by the numbers. Horizontal bar: 10 ms; vertical bar: 1 mV. **b**, HFS (200 Hz; arrow) induced reliable LTP in acclimatized animals ($n = 9$; $163.4 \pm 9.9\%$ of baseline at 60 min; $P < 0.01$ compared with pre-HFS baseline). **c**, Stable LTD was induced by LFS in unhandled, recording-naïve animals when placed for the first time in a brightly lit recording box ($n = 7$; $84.8 \pm 4.9\%$ of baseline at 30 min and $89.2 \pm 4.2\%$ 60 min after LFS; $P < 0.01$). Inset: field potentials from one experiment. **d**, HFS failed to induce potentiation of the EPSP in unhandled, recording-naïve animals when placed for the first time in the brightly lit recording box ($n = 4$; $102.6 \pm 2.2\%$ of baseline at 60 min after the tetanus). **e**, There was no baseline change between recording in the home cage (H) and the unfamiliar recording box in recording-naïve animals ($n = 8$; $P > 0.05$).

In the second stress-evoking protocol, recording-acclimatized animals were placed on an elevated platform for 30 min⁹. Behavioural 'freezing', defecation and urination were evident for the first 10 min while on the platform, but not on subsequent placement of the rat in the familiar recording box. Raised serum corticosterone was also produced by this protocol (see Methods). Homosynaptic LTD was induced in these rats when LFS was applied 5 min after removal from the elevated platform (Fig. 2a; see also Fig. 3a). Such LTD was very stable when monitored over several days (LTD on day 1 to $81.8 \pm 3\%$ versus 79.5 ± 3.8 on days 2–8; $n = 7$). Stable recordings were obtained over an equivalent period in acclimatized control animals (for example, $101.6 \pm 3.8\%$ of day 1 baseline on day 11; $n = 5$).

In the third stressful protocol, previously acclimatized (to both

handling and the recording box) animals were left without being handled in their home cage for two weeks (a period equivalent to the post-surgery acclimatization interval) and were then placed back in the recording box. This produced behavioural signs of stress similar to those that were observed on first exposure to the box. Stable LTD was elicited by LFS in these animals also (Fig. 2b).

Two other related, but non-stressful, conditions were also examined as controls. There were no behavioural or corticosterone signs of stress in acclimatized animals placed in an unfamiliar, dimly lit box, or in unhandled rats from which recordings were made for the first time in a familiar environment, their home cage. LFS failed to induce LTD in either of these situations (Fig. 2c,d).

The experimental finding that LTD could be induced in three

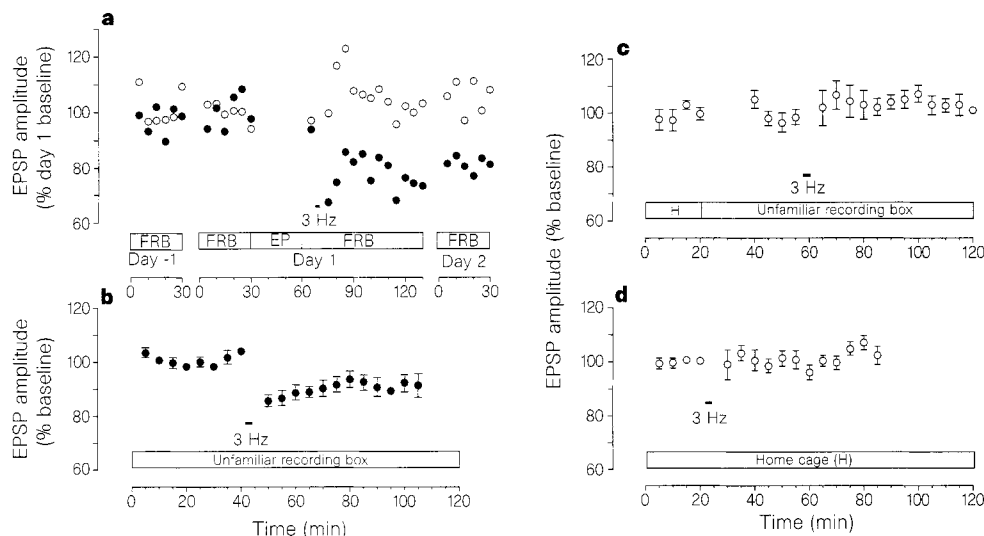


Figure 2 Presence of LTD induction in previously acclimatized animals after stress and lack of LTD induction in non-stressed animals. **a**, Placing acclimatized animals on an elevated platform (EP) for 30 min and applying LFS (3 Hz; bar) 5 min after being replaced in the familiar recording box (FRB) resulted in LTD. The example shows a 3-day experiment in which LTD was monitored up to 24 h after the LFS to the test pathway (black circles). There was no heterosynaptic LTD in the control (white circles) pathway. **b**, Resensitizing previously acclimatized

animals to the recording box by leaving them undisturbed in their home cage for 14 days resulted in LTD induction following LFS ($n = 6$; $91.6 \pm 2.7\%$ of baseline 30 min after LFS; $P < 0.05$). **c**, LFS failed to induce LTD in acclimatized rats when the experiment was carried out in an unfamiliar, dimly lit (relatively non-stressful) recording box ($n = 5$; $104.3 \pm 6.1\%$ of baseline 30 min after LFS; $P > 0.05$) (H, home cage). **d**, LFS failed to induce LTD in recording-naïve rats in their home cage ($n = 7$; $100.7 \pm 3.3\%$ of baseline 30 min after LFS; $P > 0.05$).

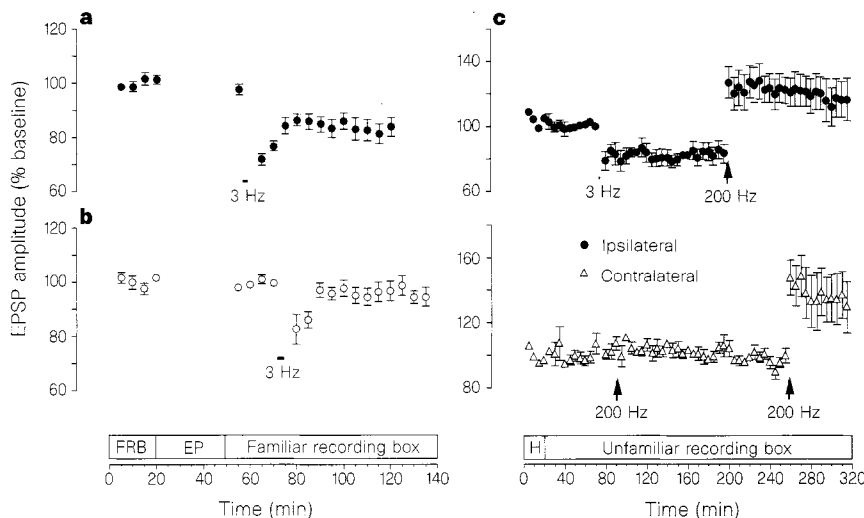


Figure 3 A narrow time window exists for the effect of stress on LTD and LTP induction. **a**, Placing acclimatized animals on the elevated platform (EP) for 30 min and applying LFS (3 Hz; bar) 5 min after being replaced in the familiar recording box (FRB) resulted in LTD ($n = 5$; $85.1 \pm 2.9\%$ 30 min after LFS; $P < 0.05$). **b**, When LFS was given 20 min after acclimatized animals were removed from the elevated platform, only STD was induced by LFS ($n = 5$). **c**, In recording-naïve animals, LTD

was induced in the test, ipsilateral, input (black circles; to $83.0 \pm 6.1\%$ of baseline, 2 h post-LFS, $n = 5$) without affecting responses in a control, contralateral, input (white triangles). High-frequency stimulation (HFS; 200 Hz; arrow in three of the 5 animals) to the control pathway 10 min later failed to induce LTP. When HFS was applied 2 h (test pathway) or 2.5 h (control pathway) later, LTP was induced (H, home cage).

stress-evoking protocols but not in three related non-stressful conditions reveals that stress itself is the important factor controlling the ability of LFS to elicit LTD in the present experiments. The lack of LTD after LFS applied either to non-acclimatized rats in their home cage or to acclimatized rats in a new non-stressful environment, taken together with the homosynaptic nature and long duration of LTD, is strong evidence against other explanations (see Methods).

Experiments to investigate the time window for LTD induction after exposure to the stress-inducing conditions revealed that it lasted for a relatively short period. In the case of the stress-inducing protocol in which acclimatized animals were placed on the elevated platform (second stressful protocol), only an LFS-induced short-term depression (STD) was observed when there was a 20-min, as opposed to 5-min (see above and Fig. 3a), interval between

replacement of the animal in the recording box and the LFS (Fig. 3b); that is, there was a loss of ability to induce LTD. There was also a rapid loss of ability to induce LTD on acclimatization to the unfamiliar recording box in recording-naïve animals (first stress protocol). Application of the LFS 95 min (as opposed to up to ~50 min; see above) after the rats were placed in the new recording box did not induce significant LTD, although an STD lasting for 5–10 min was observed ($n = 4$; $94.9 \pm 7.4\%$ of baseline at 30 min; $P > 0.05$). The loss of the ability to induce LTD in recording-naïve rats was accompanied by the recovery of the ability to elicit LTP. HFS induced significant LTP when it was applied 95–115 min after recording-naïve rats were placed in the recording box ($n = 8$; $138.9 \pm 12.5\%$ of baseline at 60 min after the tetanus; $P < 0.05$). Similarly, in recording-naïve animals in which LTD had been induced previously, the application of HFS 180 min after the rats were placed in the recording box induced LTP (Fig. 3c).

Experiments on pentobarbitone-anaesthetized animals showed that the behaviourally stressed animals did not need to be awake at the time when the LFS was applied for LTD to be elicited. When animals were anaesthetized immediately after being placed on the raised platform for a period of 30 min and LFS was applied 80–210 min later, stable homosynaptic LTD was elicited (Fig. 4a). The LFS only induced a transient heterosynaptic depression of the contralateral, commissural input. Furthermore, subsequent HFS to the control pathway failed to induce LTP in these previously stressed animals. In control, non-stressed rats, LTD was not elicited by LFS (Fig. 4b), whereas LTP was reliably induced by subsequent HFS. The lack of LTD induction is in agreement with previous studies on anaesthetized animals^{6,8,10,11} (but see ref. 12). HFS was found to reverse LTD that had been induced in previously stressed animals but did not induce LTP above the pre-LFS baseline (Fig. 4c).

Our findings reveal that behavioural stress is a key factor governing the ability of LFS to induce LTD in the awake adult animal. The period when LTD could be induced was relatively brief, being rapidly lost on acclimatization to, or removal from, the aversive conditions. Once LTD had been induced, however, it persisted for a prolonged period after this time window had elapsed. In contrast to the situation in awake animals, in which the ability to induce LTD was lost within 20 min of being removed from the elevated platform, experiments on animals that were anaesthetized immediately after removal from the platform showed that LTD could still be induced at least up to 3 h later. These results indicate that the rapid loss of ability to induce LTD in awake animals after removal from the elevated platform may be due to an active process, presumably dependent on the animal being aware of its escape from the aversive environment.

Studies on the underlying mechanisms of how stress allows LTD induction should prompt investigation into the cellular control of this form of synaptic plasticity. Given the brief time window in which LTD could be induced in awake animals, it is unlikely that corticosteroids are solely responsible, as such hormones usually have long-lasting effects¹³, although they probably have a contributory role^{14–16}. The many transmitters that are released during stress, such as amino acids, amines and peptides, could be involved. *In vitro*, relatively intense stress facilitated the induction of LTD in hippocampal slices by a mechanism that was dependent on *N*-methyl-D-aspartate (NMDA) receptor activation¹⁷, suggesting that this type of stress was acting by inducing LTP¹⁷. However, our results show that exposure to a novel stressful environment does not induce LTP while still enabling LTD to occur *in vivo*. Our results are consistent with the idea that the ability to induce either LTD or LTP can be changed without affecting baseline transmission¹⁸.

Although LTD is a complementary form of synaptic plasticity to LTP, in the intact hippocampus standard LFS-induced LTD could only be observed in our experiments under diametrically opposite behavioural conditions to those required for LTP induction; that is, stress enabled LTD and blocked LTP, whereas adaptation to stress

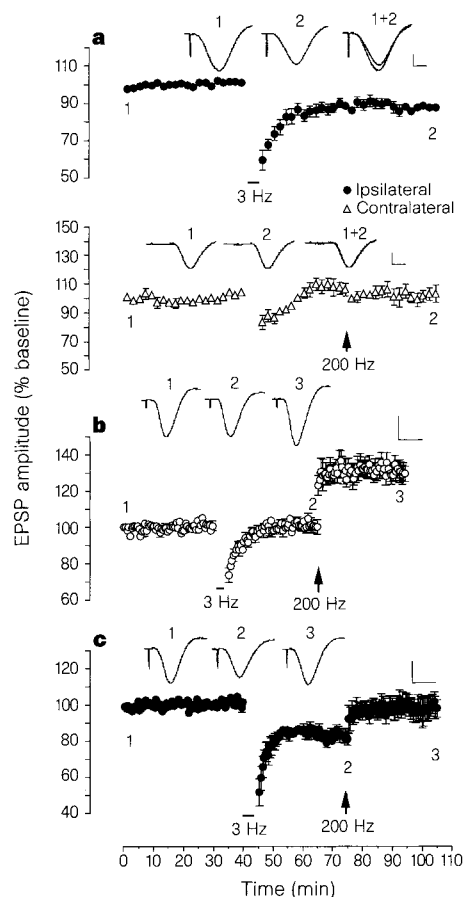


Figure 4 Previous stress allowed the induction of homosynaptic LTD by LFS in anaesthetized rats that was reversed by subsequent HFS. **a**, Stable homosynaptic LTD was induced by LFS (3 Hz; bar) in rats that had spent 30 min on an elevated platform before being anaesthetized with pentobarbitone (60 mg kg^{-1} i.p.). This LFS induced LTD in the test, ipsilateral, input (black circles; $n = 5$; $91.2 \pm 3.2\%$ of baseline 30 min after LFS; $P < 0.05$) without inducing LTD in a control, contralateral, input (white circles). HFS (200 Hz; arrow) applied 30 min later to the control pathway did not induce LTP ($105.7 \pm 3.9\%$, 30 min post-LFS). **b**, LFS only induced STD, not LTD, in anaesthetized rats that had not been placed on the elevated platform ($n = 7$; $100.3 \pm 4.0\%$ of baseline 30 min after LFS; $P > 0.05$). Subsequent HFS (200 Hz; arrow) induced LTP ($130 \pm 5.2\%$ of baseline 30 min after HFS; $P < 0.05$). **c**, When HFS was applied 30 min after the LFS in previously stressed anaesthetized animals, the LTD was reversed ($n = 5$; from $83.0 \pm 3.4\%$ of baseline 30 min after LFS to $98.1 \pm 6.2\%$ of baseline 30 min after HFS; $P < 0.05$). Insets: field potentials (average of 4 consecutive sweeps) from typical experiments at the times indicated by the numbers. Horizontal bar: 10 ms; vertical bar: 1 mV.

had the opposite effect. Therefore, if homosynaptic LTD and LTP are to operate together to allow activity-dependent modification of the stimulus selectivity of neurons⁴, this is not likely to occur concomitantly under behavioural conditions such as those examined here. As HFS is shown here to reverse LTD to pre-LFS in stressed animals, and LFS has been reported to reverse LTP in non-stressed rats^{7,8,19}, such a role for bidirectional plasticity is possible, but with a dynamic range tightly restricted by behavioural state. □

Methods

Electrode implantation. Male Wistar rats, weighing 200–300 g, had electrodes implanted using previously described techniques^{19–21}. The surgery was carried out under pentobarbitone sodium (60 mg kg⁻¹, i.p.) anaesthesia, and core temperature was maintained at 37 ± 0.5 °C. Three stainless-steel screws (1.5 mm diameter) were inserted into the skull through a drill hole without piercing the dura. One served as a ground electrode (7 mm posterior to bregma and 5 mm left of the midline), another acted as an anchor (opposite the ground screw, 7 mm posterior to bregma and 5 mm right of the midline) and the third served as the reference electrode (8 mm anterior to bregma and 1 mm left of the midline). Recording and stimulating electrodes were made by gluing together a pair of twisted Teflon-coated platinum (90%)/iridium (10%) wires (50 µm inner diameter, 75 µm outer diameter). The recording electrode was inserted 3.4 mm posterior to bregma and 2.5 mm right of the midline and the stimulating electrode was inserted 4.2 mm posterior to bregma and 3.8 mm right of the midline. In some animals, a second stimulating electrode was placed in the contralateral hemisphere in order to stimulate the commissural pathway. Lack of paired-pulse interaction with responses evoked in the test pathway was used as a criterion of independence. The optimal placement of the electrodes in the stratum radiatum of the CA1 region of the dorsal hippocampus was determined using electrophysiological criteria and was verified by post mortem examination.

Acclimatization. After surgery animals were housed individually (in home cage) with free access to water and food in an established animal house having a 12 h light/dark cycle and thermoregulated environment. Recording began two weeks later to allow the animals to recover from surgery. Acclimatized group: during this recovery period the rats were put in the brightly lit recording box (34 × 24 × 24 cm) for 1 h at the same time each day and the socket assembly on the animal's head was connected to a preamplifier by a flexible wire. By the end of the first week, there was no evidence of behavioural 'freezing' and the rats became very tame and well used to being handled. Unhandled, recording-naïve group: the rats were left undisturbed in their home cage during the two-week recovery period.

Electrophysiology. Test field EPSPs were evoked by stimulating with a square-wave constant current pulse of 0.2 ms duration at a rate of 0.033 Hz. At the beginning of each experiment, input–output curves were generated to determine the maximum field EPSP amplitude. Results were similar if the EPSP slope rather than the EPSP amplitude was measured. During the experiments, the stimulus intensity was set at a level that evoked a field EPSP amplitude of 50–60% of the maximum. LFS consisting of 900 pulses at 3 Hz was applied to induce LTD; LTP was induced by HFS (20 pulses at 200 Hz, repeated 10 times at a 2-s interval). The background EEG (monitored from the hippocampal recording electrode) showed no evidence of seizure activity after the conditioning stimulation. To minimize the possibility that changes in locomotor activity might affect the field EPSP amplitude, recordings were not usually taken for the first 20 min after being placed in the recording box unless otherwise stated. Control experiments showed that placement of either recording-naïve animals in a brightly lit unfamiliar recording box (stressed) or recording-acclimatized animals in an unfamiliar dimly lit recording box (non-stressed) was associated with a similar small (<1 °C) transient (<10 min) increase in brain temperature (recorded with a precalibrated type-T thermocouple wire (tip diameter, 0.5 mm; accuracy, 0.05 °C) placed on the surface of the contralateral hippocampus or cortex). Serum corticosterone was determined using HPLC. Levels were raised in stress conditions compared to non-stress conditions (recording-naïve/unfamiliar recording box, 18.2 ± 5.3; acclimatized/elevated platform, 40.6 ± 7.3; acclimatized/familiar recording box, 3 ± 0.8; acclimatized/novel recording box, 5.2 ± 1.2; recording-naïve/home cage 1 ± 0.3 µg dl⁻¹; n = 4 per group). Statistical comparisons between base-

line and post-conditioning stimulation values were made using Student's *t*-test. Values are mean per cent of the baseline field EPSP amplitude ± s.e.m.

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Impaired learning and LTP in mice expressing the carboxy terminus of the Alzheimer amyloid precursor protein

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Proteolytic processing of amyloid precursor protein (APP) through an endosomal/lysosomal pathway generates carboxy-terminal polypeptides that contain an intact β-amyloid domain^{1–3}. Cleavage by as-yet unidentified proteases releases the β-amyloid peptide in soluble form^{4–6}. In Alzheimer's disease, aggregated β-amyloid is deposited in extracellular neuritic plaques.

Although most of the molecular mechanisms involving β -amyloid and APP in the aetiology of Alzheimer's disease are still unclear, changes in APP metabolism may be important in the pathogenesis of the disease. Here we show that transgenic mice expressing the amyloidogenic carboxy-terminal 104 amino acids of APP develop, with ageing, extracellular β -amyloid immunoreactivity, increased gliosis and microglial reactivity, as well as cell loss in the CA1 region of the hippocampus. Adult transgenic mice demonstrate spatial-learning deficits in the Morris water maze and in maintenance of long-term potentiation (LTP). Our results indicate that alterations in the processing of APP may have considerable physiological effects on synaptic plasticity.

Transgenic mice were produced by using a construct containing an APP complementary DNA fragment encoding amino acids 591 to 695, which spans the amyloid-forming portion and the carboxy terminus of the human amyloid precursor protein, cloned into the first exon of the human neurofilament *NF-L* gene and under the transcriptional control of the *NF-L* regulatory sequences⁷ (Fig. 1a). Analysis by northern blotting showed that three out of seven transgenic lines had human amyloid-derived transcripts (~900 base pairs (bp)) in their brain tissue, expressed at levels comparable to the endogenous APP gene (Fig. 1b). Furthermore, expression of the transgene was restricted to nervous tissue and followed the pattern of expression of *NF-L* (ref. 7; data not shown). The presence of multiple copies of the transgene did not disrupt *NF-L* metabolism as there was no difference in the amount of *NF-L* transcript or protein expressed in transgenic and control mice (data not shown). In immunoprecipitation experiments using an anti-C-terminus antibody⁸, brain homogenates prepared from transgenic mice had an increase of 30–50% in the levels of 12K polypeptide (relative molecular mass 12,000; Fig. 1c). Several smaller polypeptides that were immunoreactive with a polyclonal antibody against synthetic β -amyloid peptide¹ were detectable by western blotting (Fig. 1d): the pattern of bands obtained is similar to the five C-terminal APP fragments detected in human brain¹. No 4K band was seen on immunoblots or in immunoprecipitates.

Immunohistochemical analysis of brain tissue from transgenic mice revealed that, with ageing, the extracellular immunoreactivity

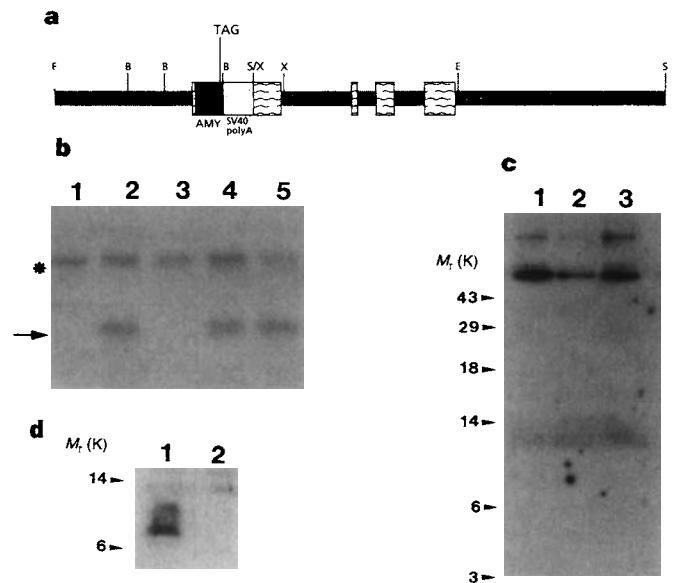


Figure 1 Expression of C-terminal amyloidogenic fragment of APP in transgenic brain. **a**, The construct: human *NF-L* gene upstream, intron and downstream genomic sequences are in grey; exon sequences are represented by white boxes. B, *Bam*HI; E, *Eco*RI; S, *Sal*I; X, *Xho*I; AMY, APP sequences; TAG, stop codon. **b**, Analysis of endogenous APP (asterisk) and transgenic mRNA (arrowed) levels in brain. Lane 1, control brain; lanes 2–5, transgenic brain. Note that no transgenic mRNA was detectable in lane 3 and the founder was not kept for further study. **c**, Immunoprecipitation of brain homogenates with an anti-C-terminus antibody⁸. Lane 1, control mouse brain; lanes 2, 3, transgenic mouse brain. **d**, Detection of amyloidogenic C-terminal fragments in brain homogenates (100 μ g) of transgenic mice (lane 1) and control mice (lane 2) after electrophoresis on Tris/Tricine–16.5% polyacrylamide gel and immunoblotting with a polyclonal anti-amyloid antibody (SGY2347)¹.

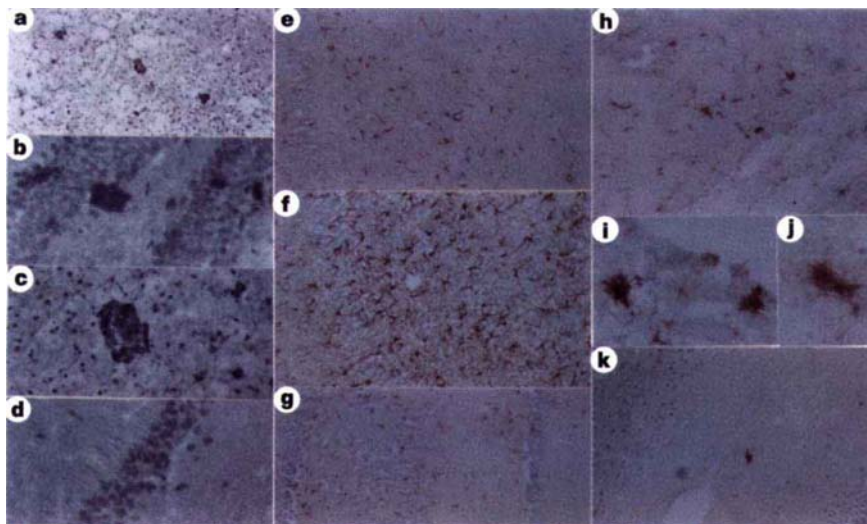


Figure 2 Immunohistochemical characterization of brain tissue from representative transgenic and age-matched control mice. Frozen sections were reacted with an anti-amyloid antibody (R1282) which revealed extracellular immunoreactivity in adult transgenic mouse cortex (**a**, **c**) and hippocampus (**b**); only intracellular reactivity was detected in hippocampus (**d**) of aged controls. Extensive gliosis was evident as anti-GFAP-positive astrocytes in the hippocampus

of aged transgenics (**f**); adult transgenics (**e**) had more reactive astrocytes than aged controls (**g**) and young controls were devoid of immunoreactivity (not shown). Microglia that were detected with the anti-Mac-1 antibody in transgenic brain (**h**, **i**, **j**) were rare in control brain (**k**). Tissue was processed as described in Methods. Original magnifications: **a**, 150 $\times$; **b–d**, **i**, **j**, 600 $\times$; **e–h**, **k**, 300 $\times$.

for β -amyloid became more prevalent. The immunoreactivity was present as dense, compact structures in both cortex and hippocampus of only transgenic mice and was absent in controls (Fig. 2a–d). They were accompanied by generalized gliosis (Fig. 2e–g), which also increased with ageing: glial fibrillary acidic protein (GFAP)-positive reactive astrocytes, absent in controls, appeared as early as 4–5 months of age in hippocampal and cortical regions of the transgenic mice. Differences in microglial reactivity were evident in transgenic mice compared to age-matched controls (Fig. 2h–k). In our aged animals, cell counts in the CA1 region of the hippocampus⁹

revealed a decrease in cell density of $\sim 20\%$ in transgenic animals ($0.295 \pm 0.06 \times 10^6$) compared to controls ($0.379 \pm 0.09 \times 10^6$) ($P < 0.001$). In this region, total neuron counts were also decreased in transgenic mice ($0.79 \pm 0.06 \times 10^4$) compared to control animals ($0.89 \pm 0.09 \times 10^4$) ($P < 0.007$).

To determine whether expression of the transgene in these animals imparted a phenotype, we characterized their behaviour in the Morris swim maze, which provides a sensitive assay for brain damage¹⁰, particularly in the hippocampal or neocortical regions^{11,12}. Mice are placed in a specific quadrant of a pool of

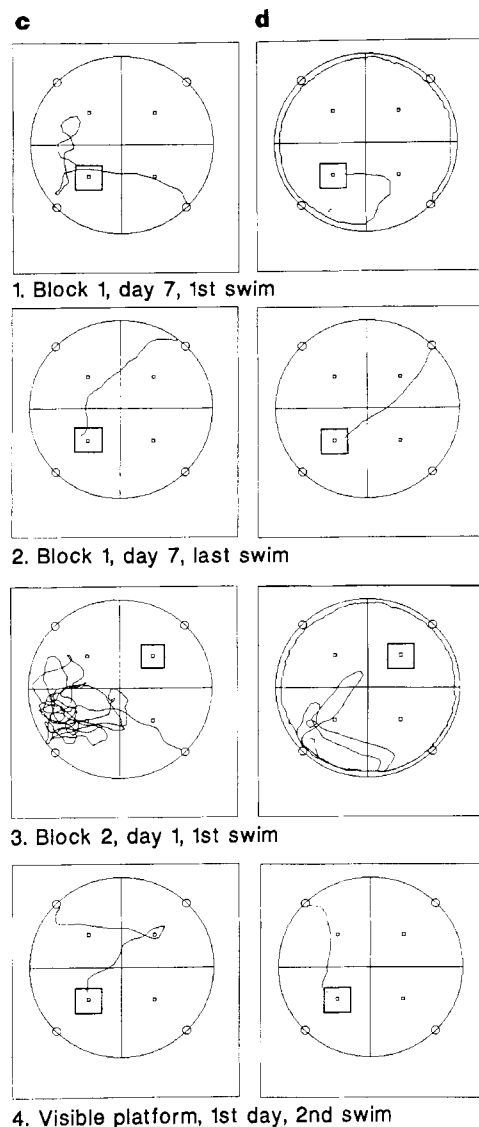
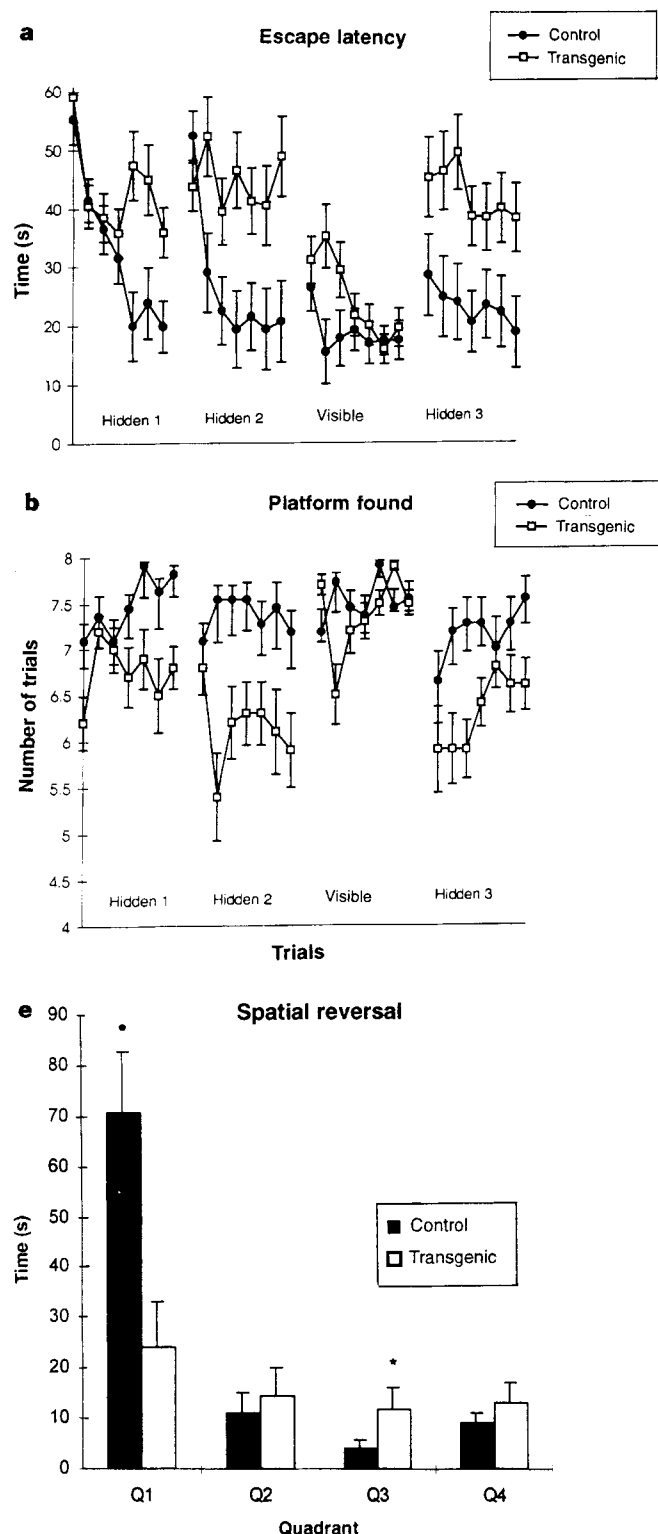


Figure 3 Performance of control (circles) and transgenic mice (squares) in the water maze was tested under four conditions, with the platform being hidden under the water in quadrants I, II and III of the tank (spatial learning), or made visible with a flag (cued learning). **a**, Escape latency. **b**, Number of trials in which mice found the platform during the 120-s trial. **c**, **d**, Examples of swim paths recorded during different stages of training for **c**, a control mouse and **d**, a transgenic mouse. Large squares show the platform locations, small squares indicate the quadrant centre, small circles the starting points, and lines the quadrants of the maze. **e**, The spatial probe trial was quantified by an ANOVA test comparing the total time spent in each of the four quadrants (Q1–4) of the maze. * $P < 0.05$.

opaque water and use spatial cues to escape to a hidden platform. Escape latency, the time required for the mice to swim to the platform, is an estimate of spatial learning and memory. To ensure that we used an unbiased measure of performance, the genetic background of the mice was coded and was unknown to the tester during the assessment. A trial began as soon as the mouse entered the water and ended as soon as it reached the platform or when 120 s had elapsed, whichever came first. Control adult mice learned to escape to the hidden platform in ~ 20 s after 5 days of testing in the first hidden platform condition (Fig. 3a and c2). The control mice performed equally well when the platform had to be found by visual guidance rather than spatial memory (Fig. 3a). In contrast, although transgenic mice improved on the hidden platform trials (Fig. 3a, d2), they were slower to find the hidden platform than control mice (3-way ANOVA (group, trial type, day) $F(18,342) = 2.84$, $P < 0.001$), and the impairment was significant in the first two tests of spatial learning (d.f.(1,19) = hidden 1, $F = 3.24$, $P < 0.05$; hidden 2, $F = 4.2$, $P < 0.05$, both 1-tailed) (Fig. 3a). In the visible platform trials, transgenic mice learned normally and no performance differences existed by the final 4 days of cued learning

(visible, $F(1,19) = 0.14$, $P = 0.71$) (Fig. 3a, b). Furthermore, whereas the control mice typically found the platform within the allotted 120 s in every trial, the transgenic mice found the hidden platform within the allotted time significantly less frequently than the control mice during the last three trials in hidden 1 and hidden 2 ($F(1,19) \geq 3.0$, $P \leq 0.05$, 1-tailed), but they found the visible platform normally ($P = 0.99$) (Fig. 3b). There were no differences in swimming abilities as the transgenic mice swam faster but with less spatial accuracy than the controls; on average, transgenic mice swam longer distances, at a greater mean distance from the platform (data not shown). Transgenic mice did not differ from controls in general locomotor activity, as measured by interruptions of photocell beams in activity boxes equipped with photocell detectors (data not shown).

Examples of swim paths recorded during different stages of training for a control mouse and a transgenic mouse are shown in Fig. 3c, d. When the platform was moved to a different location (Fig. 3, c3 and d3), control mice swam persistently over the former platform location and thus took longer to find the platform in the reversal trial than the transgenic mice, whose more random swimming pattern meant that they intersected more quickly with the new platform location (Fig. 3a, hidden 2, first swim). The spatial bias of control mice (Fig. 3, c3) and the absence of such bias in transgenic mice (Fig. 3, d3) were confirmed statistically (interaction of quadrant by group: $F(3,57) = 10.8$, $P < 0.001$) (Fig. 3e). In contrast to transgenic mice, control mice spent more time in the first quadrant (Q1) than in the other quadrants (effect of quadrants: controls only, $F(3,30) = 26.8$, $P < 0.001$; transgenic only, $F(3,27) = 1.4$, $P < 0.27$). Control mice spent more time than the transgenic mice in Q1 ($F(1,19) = 10.3$, $P < 0.005$), whereas transgenic mice spent more time than the control mice in the opposite quadrant ($F(1,19) = 3.2$, $P < 0.05$ (1-tailed)) (Fig. 3e). Trials with the visible platform, in which transgenic mice swam directly towards the visible platform (Fig. 3, d4) indicated that the thigmotactic behaviour shown early in spatial trials (Fig. 3, d1 and d3) was a consequence of impaired spatial learning, rather than a general alteration in behavioural strategy. These results indicate that the transgenic mice are selectively impaired in spatial learning but unimpaired in cued (visual discrimination) learning.

A model system for studying storage of memory and learning is through assay of synaptic plasticity, which can be induced by a short burst of high-frequency stimulation, a process known as long-term potentiation¹³. As shown in Fig. 4a, synaptic potentiation in CA1 was induced by theta burst stimulation (TBS) in hippocampal slices of control and transgenic mice. There was no significant modification in the degree of short-term potentiation in the first 2 min following TBS in transgenic mice ($58 \pm 6\%$; $n = 10$) compared to control mice ($66 \pm 7\%$; $n = 10$), but for the transgenic mice, there was a progressive decay of long-term potentiation (LTP) with time. A significant reduction in the magnitude of potentiation was found 10 min after TBS ($P < 0.05$, $n = 10$; Tukey test) and an even greater reduction after 50 min ($52 \pm 4\%$ in control compared with $15 \pm 4\%$ in transgenics). There was a similar reduction in a second independent transgenic line (data not shown). The degree of paired-pulse facilitation measured in transgenic mice ($40 \pm 5\%$, $n = 5$) was not significantly modified when compared to that measured in control mice ($45 \pm 7\%$, $n = 7$). As paired-pulse facilitation is generally thought to be due to presynaptic enhancement of transmitter release, it may be assumed that presynaptic function is not altered in transgenic animals.

Long-term depression (LTD) is a second major form of activity-dependent synaptic plasticity¹⁴. LTD and LTP share many cellular substrates, but in LTD, low-frequency stimulation leads to a decrease in synaptic response. There were no differences in the degree of homosynaptic LTD between control and transgenic mice (Fig. 4b). These results demonstrate that whereas LTP maintenance is diminished in transgenic mice, normal LTD can be generated.

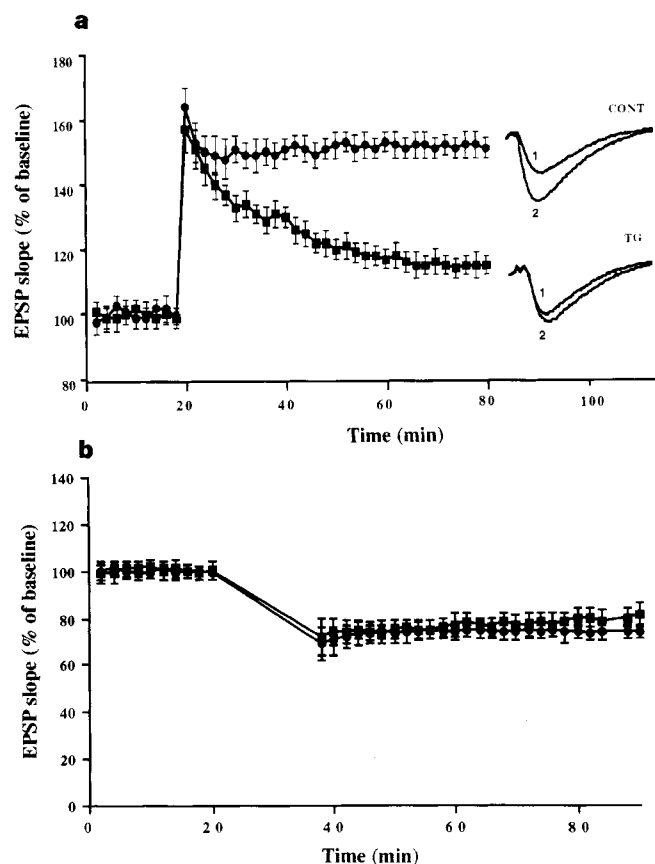


Figure 4 Reduced LTP magnitude (a) but normal LTD formation (b) in hippocampus of transgenic (squares) as compared to control mice (circles). **a**, LTP was measured in slices prepared from control ($n = 6$) and transgenic mice ($n = 6$). The initial slope of each excitatory postsynaptic potential (EPSP) evoked by electrical stimulation (every 30 s) in area CA1 of the hippocampus was calculated and the values of four successive responses were averaged. The baseline value represents the average of all the values obtained during the 20-min interval preceding theta burst stimulation. Data are expressed as percentage of the baseline value and each point represents the mean $\pm$ s.e.m. of 10 different experiments. Records were taken before (1) and 50 min after (2) theta burst stimulation. **b**, LTD was initiated by low-frequency stimulation (900 pulses at 1 Hz). Data are expressed as a percentage of the baseline value and each point represents the mean $\pm$ s.e.m. of 10 different experiments.

Table 1 Characteristics of transgenics expressing different isoforms/fragments of APP

Construct	Extracellular amyloid	Neuronal loss	Behavioural deficits		Electrophysiological alterations	Ref.
			Spatial	Cued		
APP (Val 717 → Phe)	+	ND	ND	ND	ND	28
APP ₇₅₁ (WT)	±	ND	+	–	ND	29
APP ₆₉₅ (Lys 670 → Asn; Met 671 → Leu)	+	ND	+	+	ND	27
APP (C104)	+	+	+	–	+	This work

ND, not determined. WT, wild type.

* Transgenic mice were also impaired in the visible platform test.

To determine whether a reduction in NMDA (*N*-methyl-D-aspartate) receptor number and/or function might impair LTP in transgenic mice, the binding of 100 nM ³H-glutamate was assayed. Autoradiography revealed no substantial changes in the amount of binding in various regions of the brain, including the CA1 stratum radiatum of the hippocampus (1.95 ± 0.15 pmol per mg protein in transgenic mice ($n = 4$) versus 2.07 ± 0.13 pmol mg⁻¹ in controls ($n = 5$)). In addition, the NMDA-mediated response evoked in the presence of low Mg²⁺ and the AMPA (α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptor antagonist DNQX (6,7-dinitroquinoxaline-2,3-dione) was normal in the transgenic mice (G.M., unpublished results). Thus, it is unlikely that a reduction in NMDA receptor function alone could account for the LTP deficit in transgenic mice.

In our transgenic mice, expression of a C-terminal amyloidogenic fragment led to tissue injury, with gliosis and microglial activation as well as extracellular amyloid immunoreactivity and hippocampal cell loss in the aged animals. There were marked behavioural and electrophysiological deficits in adult animals. Histological analysis and the selective changes in behaviour and electrophysiology, indicate that expression of human C-terminal APP in the brains of transgenic mice does not lead to massive and outright neuronal cell death as it does in neuronal cell lines transfected with constructs bearing this fragment^{15,16}. Our results show that chronic over-expression of the amyloidogenic C-terminal fragment of APP is important physiologically. In Down's syndrome, duplication of the APP gene on chromosome 21 seems to be sufficient for amyloid deposition and subsequent development of Alzheimer-type neuropathology¹⁷, so increased expression of the APP gene may alter the processing of the protein, producing more insoluble β -amyloid. *In vitro* studies with synthetic peptide have ascribed many neurotoxic effects to the aggregated form of β -amyloid^{18–21}, with the effects being mediated by reactive oxygen species²¹, by alterations in intracellular calcium metabolism¹⁹, or by interference with potassium²² and calcium channels²³. β -Amyloid can also activate microglia^{24–26}, generating free radicals which may damage neighbouring neural cells and initiate an inflammatory loop with the release of cytokines, locally increased levels of β -amyloid, and further activation of microglia. Varying degrees of extracellular amyloid immunoreactivity and some associated behavioural deficits (occurring as a function of genetic background²⁷) have been reported in transgenic mice expressing different variants of APP (Table 1). Our discovery of early physiological perturbations that occur in transgenic mice expressing the amyloidogenic C-terminal fragment of human APP have important implications for the pathogenesis of Alzheimer's disease. □

Methods

Transgene construct. A *Bgl*II/*Pvu*II fragment (nucleotides 1,768 to 2,223) from the 3'-end of human APP cDNA³⁰ (encoding amino acid 591 to the termination codon) was cloned into the plasmid pSVL (Pharmacia). A *Sma*I/*Sa*II fragment containing the APP cDNA, as well as the SV40 late polyadenylation signal from pSVL, was cloned into a plasmid containing the entire human *NF-L* gene which had been digested by *Sma*I and partially by *Xho*I. Insertion of the APP clone was verified by sequencing. The entire 11.5-kb

fragment was microinjected after removal of backbone plasmid sequences into fertilized oocytes from B6C3 F₁ mice.

Immunohistochemistry. Adult (8–10 months) or aged (18–22 months) mice were anaesthetized by intraperitoneal injection of chloral hydrate and perfused through the heart with a 4% solution of buffered paraformaldehyde. The brain was postfixed for 1 h with 4% buffered paraformaldehyde, and cryoprotected with 20% sucrose before freezing at -50°C and storage at -70°C . Cryostat sections (15 μm thick) from transgenic and control animals were processed in parallel. Sections were treated with 88% formic acid for 5 min before assaying for anti-amyloid immunoreactivity with R1282 (diluted 1:1,000; provided by D. Selkoe) using a Vectastain ABC (Dimension Labs) kit for rabbit polyclonal antibodies. The signal from horseradish peroxidase was revealed with the enhanced diaminobenzidine substrate (Pierce). A polyclonal anti-cow GFAP antibody (Dako) that reacts strongly with the mouse GFAP was used at a dilution of 1:10,000 and the signal was revealed with the horseradish peroxidase substrate aminoethylcarbazol which produces an insoluble red product. Microglial reactivity was detected with anti-Mac-1 antibody (1:9) (prepared from the supernatant of the rat hybridoma M1/70.15.11.5.HL; obtained from the American Type Culture Collection). This antibody reacts against the I-subunit of Mac-1 antigen (complement receptor type 3) in mice and humans. An anti-rat secondary antibody was used to reveal specific staining with aminoethylcarbazol. Sections were counterstained with haematoxylin.

Cell counting. Neurons were counted on cryostat frozen 15- μm sections stained with cresyl violet prepared from aged transgenic ($n = 6$) and non-transgenic animals ($n = 12$). The entire CA1 region was delimited using a computer-assisted image analysis system (Biocom, France) which calculates areas automatically, and cells were counted for both hippocampi on each section ($n = 4$)⁹. Results were expressed as neuronal density (neurons per mm³ $\pm$ s.d.). The transgenic population was considered to be homogeneous as the distribution of neuronal density was not different within the group (Kruskal–Wallis non-parametric analysis of variance test). Neuronal counts were obtained by serial sectioning of a segment spanning 250 μm of dorsal CA1. The Mann–Whitney non-parametric analysis of variance test was used to compare data obtained with transgenic and control mice.

Behavioural testing. Mice tested consisted of eight-month-old animals transgenic for the C-terminal fragment of APP ($n = 10$) or for the prokaryotic gene chloramphenicol acetyltransferase driven by the same human *NF-L* sequences ($n = 11$). Both lines of mice had been produced in the same transgenic mouse facility. The experimenter was blind to the genotype of the mice. The maze (170 cm in diameter and 70 cm of height) was lined with white coloured plastic and filled with a 45-cm column of opaque water (20°C). For the testing phase, rectangular cues (22 $\times$ 28 cm) with differing geometric patterns were placed at 3 cm above the water at each of four locations between the quadrants. A video camera above the maze recorded the path swum by each mouse, and the path was stored by computer for further analysis. A training phase was administered to the mice to assess whether the animals could swim and whether they could climb onto the platform. Testing began the day after successfully determining that all test animals could swim to and climb onto the platform eight consecutive times. During the testing phase, each mouse was placed in the water facing the wall of the tank at one of the four starting points located at the centre of each quadrant. If a mouse did not climb onto the platform by the end of a maximum period of 120 s, it was placed onto the platform by hand. Each mouse was given eight swims a day. During testing carried out with the visible platform, the platform was moved to a new location

every day. A 3-way analysis of variance (ANOVA) compared the behaviour of the two groups across days and trial types.

Electrophysiological studies. Experiments were done on 450- μ m-thick hippocampal slices prepared from control and transgenic mice. Slices were maintained at 35°C in an artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 5 mM KCl, 1.25 mM Na_2PO_4 , 1.5 mM MgCl_2 , 2.5 mM CaCl_2 , 26 mM NaHCO_3 and 10 mM glucose pH 7.4. They were exposed to a humidified atmosphere of 95% O_2 /5% CO_2 and perfused continuously at a flow rate of 1 ml min⁻¹. After a 1-hour equilibration, a glass recording electrode (1–5 m Ω) filled with 2 M NaCl was positioned in the stratum radiatum of area CA1 of the hippocampus to record population EPSPs evoked by a bipolar electrode activating fibres of the Schaffer commissural system. Bipolar stimulating electrodes were placed in the stratum radiatum of two independent pathways (Schaffer commissural pathways) converging on common postsynaptic target cells. The independence of each pathway was confirmed by the absence of heterosynaptic LTD or LTP in the pathway not receiving tetanic stimulation. After a 20-min baseline period, during which responses were recorded every 30 s, a low-frequency stimulation train of 1 Hz for 15 min (900 pulses) was applied to generate LTD; to increase presynaptic input activity, the duration of the stimulus was extended from 0.1 to 0.2 ms during low-frequency stimulation. LTP was produced by applying a high-frequency train consisting of high-frequency bursts (4 pulses, 100 Hz) repeated at 200 ms, the frequency of the hippocampal theta rhythm. The response to stimulation was quantified by calculating the initial slope of the resulting EPSP. The degree of paired-pulse facilitation was determined at an interpulse interval of 50 ms ($n = 5$ –7) and data were expressed as a percentage increase in the amplitude of the second EPSP compared to the first.

Binding studies. Adjacent horizontal and coronal cryostat sections (14 μ m) were preincubated for 60 min in 100 mM Tris-acetate buffer (pH 7.4) containing 100 μ M EGTA at 0°C. After a rapid wash in ice-cold Tris-acetate, sections were incubated with 100 nM ³H-glutamate (51 Ci mmol⁻¹; NEN-Dupont) for 45 min at 4°C in 50 mM Tris-acetate (pH 7.4), 50 μ M EGTA, 5 μ M AMPA, 1 μ M kainic acid and 10 μ M quisqualate to eliminate glutamate binding to non-NMDA sites, and 100 μ M SITS (4-acetadino-4'-isothiocyanato-stilbene-2,2'-disulphonic acid) to block glutamate-uptake sites. Non-specific binding was defined as that measured in the presence of 1 mM glutamate. After washing, dried sections, as well as tritium standards (ARC), were exposed to tritium-sensitive film (Hyperfilm, Amersham) for 14 d. Optical densities of different brain regions were converted to radioactive units using the tritium standards after image analysis. ANOVA was followed by Scheffé post hoc test, with statistical significance set at $P < 0.05$.

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Distinct ATP receptors on pain-sensing and stretch-sensing neurons

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The initial pain from tissue damage may result from the release of cytoplasmic components that act upon nociceptors, the sensors for pain. ATP was proposed to fill this role^{1,2} because it elicits pain when applied intradermally³ and may be the active compound in cytoplasmic fractions that cause pain⁴. Moreover, ATP opens ligand-gated ion channels (P2X receptors) in sensory neurons^{5,6,7} and only sensory neurons express messenger RNA for the P2X3 receptor^{8,9}. To test whether ATP contributes to nociception, we developed a tissue culture system that allows comparison of nociceptive (tooth-pulp afferent) and non-nociceptive (muscle-stretch receptor) rat sensory neurons. Low concentrations of ATP evoked action potentials and large inward currents in both types of neuron. Nociceptors had currents that were similar to those of heterologously expressed channels containing P2X3 subunits, and had P2X3 immunoreactivity in their sensory endings and cell bodies. Stretch receptors had currents that differed from those of P2X3 channels, and had no P2X3 immunoreactivity. These results support the theory that P2X3 receptors mediate a form of nociception, but also suggest non-nociceptive roles for ATP in sensory neurons.

In primary cultures of sensory neurons, nociceptors coexist with neurons that transduce other sensations. Although many *in vitro* techniques identify subpopulations of these neurons, no method identifies sensory modality^{10,11}. The inability directly to compare neurons that mediate nociceptive and non-nociceptive sensations confounds investigation of the role of ATP in specific sensations

such as pain. Here we demonstrate techniques that fluorescently label populations of nociceptors and muscle-stretch receptors. These preparations allowed us to identify properties unique to nociceptors.

Identification of nociceptors stems from the observation that application of any physiological stimulus—thermal^{12,13}, osmotic¹⁴, chemical¹⁵ or mechanical¹²—to the human tooth pulp produces pain, and only pain. By this criterion, virtually all tooth-pulp afferents are defined as nociceptors. Nociceptors were labelled in tooth pulp *in vivo* by placing a fluorescent dye (DiIC₁₈) that was

transported to somata in the trigeminal ganglia¹⁶. Tooth-pulp nociceptors are heterogeneous. Like skin, the tooth pulp is innervated by both unmyelinated C fibres and myelinated A fibres which mediate dull, diffuse pain and sharp, shooting pain, respectively^{15,17,18}.

Identification of muscle-stretch receptors relies on the unusual localization of sensory neurons to a region of the brainstem called the mesencephalic nucleus of the fifth nerve (MeN5). MeN5 sensory neurons consist of only two modalities: periodontal mechanoreceptors and jaw-muscle-stretch receptors^{19,20}; both are non-nociceptive. Dye placed in jaw muscle labels stretch receptors.

Does ATP directly excite nociceptors? As extracellular ATP degrades quickly *in vivo*²¹, we simulated physiological levels by applying a concentration 500-fold lower than that found inside cells. In a typical labelled nociceptor (Fig 1a,b), 10 μ M ATP evoked a train of action potentials whose frequency diminished during application (Fig. 1c). Subsequent application evoked little response

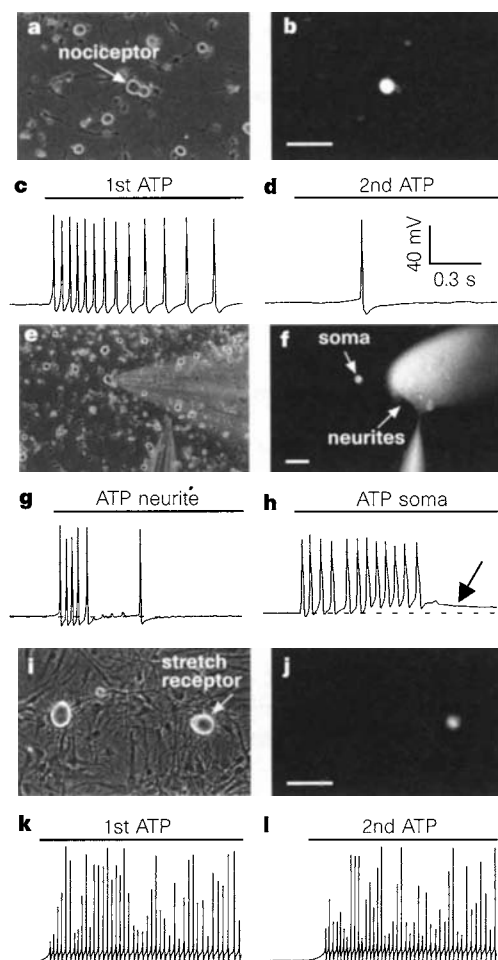


Figure 1 ATP (10 μ M, 1 s) excited both nociceptive and non-nociceptive sensory neurons. **a**, Corresponding phase micrograph, and **b**, fluorescence micrograph of a nociceptor in primary culture. **c**, ATP applied by puffer pipette to a labelled nociceptor triggered action potentials with diminishing frequency. **d**, Response 1 min later. Eight of 12 nociceptors had this adapting response. Two others did not adapt and 2 responded to ATP with just a single action potential. **e**, Phase micrograph, and **f**, fluorescence micrograph of a nociceptor, and recording and application pipettes. ATP application to neurites was visualized by the inclusion of fluorescent dye; bath solution flowed rightward. **g**, Response to neuritic application. **h**, Response to somal application included a sustained depolarization (arrow). **i**, Phase, and **j**, fluorescence micrographs of a labelled muscle-stretch receptor. **k**, Action potentials of stretch receptors did not adapt. **l**, Response 1 min later. Sampling rate (1 ms) was insufficient to capture all action potential peaks in **k**, **l**. In **c**, **k**, ATP application was 5 min after ATP. Scale bars: 100 μ m.

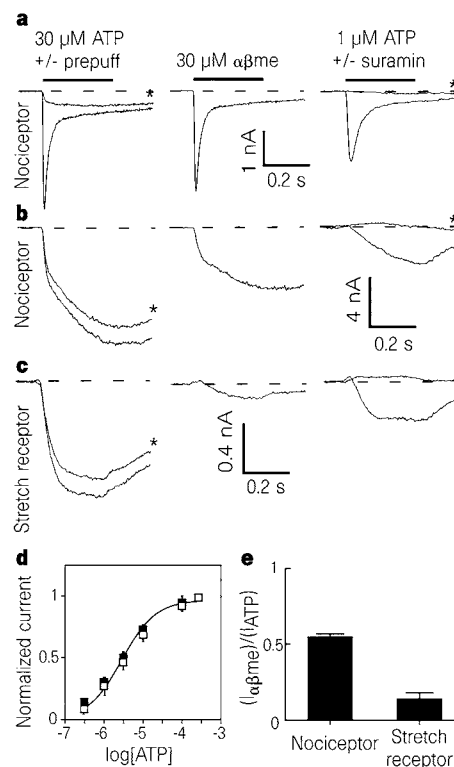


Figure 2 Pharmacological comparison of ATP-activated currents in nociceptive and non-nociceptive (MeN5) sensory neurons. Nociceptors express two types of currents and MeN5 neurons only one. **a**, Transient current in nociceptors peaked and declined during ATP application (30 μ M, 0.3 s) and was greatly diminished by prior (15 s) ATP (prepuff; asterisk). This current was activated by α , β -methylene-ATP (30 μ M), and blocked by suramin (asterisk), a P2X antagonist (10 μ M suramin, 1 μ M ATP). **b**, Persistent nociceptor current. **c**, Stretch-receptor current. **d**, ATP dose-response curves for persistent currents in nociceptors (black squares; $n = 6$) and MeN5 neurons (white squares; $n = 8$). Curve is a best fit to a single-site binding equation. **e**, 30 μ M α , β -methylene-ATP activated current in MeN5 neurons that was only 15% of that in 30 μ M ATP, compared to 55% in nociceptors ($n = 14$ MeN5 neurons; $n = 24$ nociceptors). Transient current was present in 28% of nociceptors ($n = 74$) and averaged -0.7 nA peak amplitude (1 min stimulus intervals at -80 mV). Rapid desensitization (<100 ms) and slow recovery (10 min; not shown) causes underestimation of transient current amplitude and abundance. Persistent current was present in 55% of nociceptors and averaged -1.0 nA. 16% had both currents and 19% responded only once or failed to respond. Relative abundance of transient and persistent currents did not correlate with cell body diameter. Persistent current in MeN5 neurons averaged -0.6 nA.

(Fig. 1d). Although most nociceptors showed such adaptation, some had non-adapting responses (Fig. 1 legend).

As the physiological response of sensory neurons initiates at peripheral endings and not at soma, we compared responses when ATP was applied to neurites (Fig. 1e,f) or to soma. Somal ATP application evoked smaller, broader action potentials that were superimposed upon a sustained depolarization (Fig. 1h, arrow). This sustained depolarization indicates activation of channels near the soma. Application to neurites does not cause this somal depolarization, but it triggers action potentials that then propagate to, and are detected at, the soma (Fig. 1g). Thus, neurites of cultured nociceptors respond to ATP as would occur *in vivo* during a sensory event.

Does ATP activate only nociceptors? Application of 10 μ M ATP to a stretch receptor (Fig. 1i,j) evoked high-frequency trains of action potentials that did not adapt (Fig. 1k,l; $n = 12$). Thus, extracellular ATP is not modality-selective: it activates both nociceptive and non-nociceptive sensory neurons. Patch clamp analysis of ATP-activated currents in MeN5 brain slices also suggests non-nociceptive roles for ATP in sensory neurons²².

Membrane current measurements were used to examine channels whose activation underlies the excitation of nociceptors by ATP (Fig. 2). Consistent with distinguishable adapting and non-adapting action potential responses, nociceptors ($n = 74$) have distinguishable transient (Fig. 2a) and persistent (Fig. 2b) currents. ATP-activated currents in nociceptors are large, often approaching those of voltage-dependent sodium currents that drive the action potentials.

Transient current was eliminated by prior application of ATP (prepudding), was activated by α,β -methylene-ATP, and was blocked by suramin, a P2X receptor antagonist (Fig. 2a). When heterologously expressed in human embryonic kidney cells (HEK293), P2X3 and P2X1 receptors have currents with similar properties^{9,23}. The absence of P2X1 immunoreactivity in nociceptors (see below) leaves P2X3 as the likely candidate. Persistent current also was activated by α,β -methylene-ATP, and was antagonized by suramin, but was relatively insensitive to prior application of ATP (Fig. 2b). None of the seven cloned P2X receptors expressed as homomers have this profile²⁴, but coexpression of P2X3 and P2X2 results in

similar current⁹. Hence, P2X3 receptors appear to form heteromeric channels, causing persistent current, and homomeric channels, causing transient current.

If P2X3 receptors mediate nociception, they must be present in the peripheral endings of nociceptors *in vivo*. Consistent with this requirement, antisera generated against the carboxy terminus of P2X3 identified P2X3 immunoreactivity in nerve fibres and endings in tooth pulp (Fig. 3a). In contrast, antisera that specifically recognize P2X1 (ref. 25) did not label the same tooth-pulp preparation. The accumulation of P2X3 immunoreactivity proximal to the site of infraorbital nerve crush (Fig. 3b) indicates that P2X3 is synthesized in trigeminal ganglia and is delivered to the periphery by axonal transport. In total, primarily small-, but also some large-diameter sensory neurons in the trigeminal ganglia showed P2X3 immunoreactivity (Fig. 3c). Forty per cent of retrogradely labelled nociceptors also contained P2X3 immunoreactivity (Fig. 3d,e). These results indicate that P2X3 receptors are poised to detect ATP released near sensory endings in tooth pulp.

Unlike ATP-activated currents in nociceptors, all currents in MeN5 sensory neurons ($n = 34$), including stretch receptors, were persistent. Like the persistent current of nociceptors, current in MeN5 neurons (Fig. 2c) resisted desensitization by prior ATP application, and was antagonized by suramin. It also was equally sensitive to ATP (half-maximal effective concentration, $EC_{50} = 2.9 \pm 0.3 \mu$ M; Fig. 2d). However α,β -methylene-ATP only weakly stimulated MeN5 neurons (Fig. 2e). The insensitivity to α,β -methylene-ATP indicates that P2X3 receptors do not mediate MeN5 current and is consistent with the absence of P2X3 immunoreactivity in MeN5 neurons (Fig. 3f). Instead, MeN5 current resembles currents found when either P2X2 or P2X5 receptor subtypes are expressed as homomers^{24,26}. Because mRNA for P2X5 and not P2X2

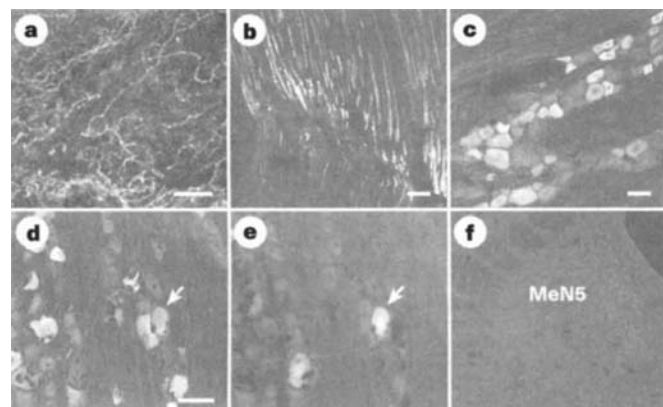


Figure 3 P2X3-immunoreactivity (ir) in nociceptors and their peripheral endings *in situ*. Antisera generated against P2X3 receptor labelled sensory endings in tooth pulp and a subpopulation of primary afferent neurons in trigeminal ganglia. **a**, P2X3-ir of nerve fibres in tooth pulp. **b**, Accumulation of P2X3-ir proximal (top) to infraorbital nerve crush. **c**, P2X3-ir was observed mostly, but not exclusively, in trigeminal neurons with small-diameter cell bodies. **d**, P2X3-positive, and **e**, Fluorogold (FG)-labelled trigeminal ganglion neurons shown in the same field of view. Arrows point to a neuron that contains both P2X3-ir and FG. **f**, P2X3-ir was absent from the MeN5, which contains sensory neurons that are strictly non-nociceptive. Scale bars: **a**, 25 μ m; **b**, **c**, **f**, 100 μ m; **d**, **e**, 100 μ m.

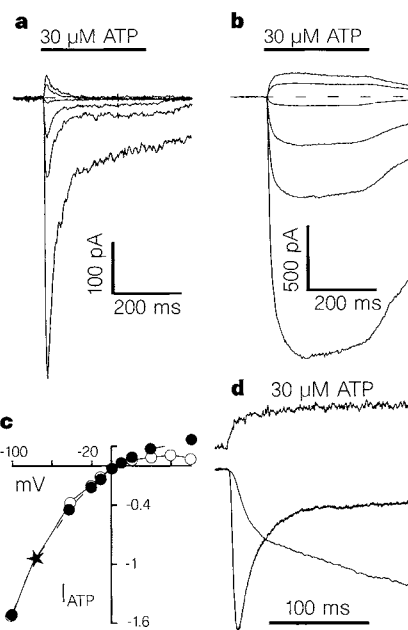


Figure 4 Physical properties of ATP-activated currents in nociceptors. **a**, Transient, and **b**, persistent responses to ATP (30 μ M, 0.3 s). Membrane voltage was held at -80 (most negative current), -40 , -20 , 0 , 20 or 40 mV (most positive). Kinetics were unaffected by voltage. **c**, Average transient (filled circles; $n = 8$) and persistent (open circles; $n = 6$) currents (normalized to value at -80 mV) versus voltage. Both channels rectify and reverse near 0 mV. **d**, Scaled transient and persistent currents from two neurons. Top record shows the measured time course of ATP application (see Methods).

is found in MeN5 (ref. 24), the P2X5 receptor probably mediates ATP-activated currents in these neurons.

The two kinetically distinct currents of nociceptors have similar permeation properties (Fig. 4). Transient (Fig. 4a) and persistent currents (Fig. 4b) reversed at 6 mV, as expected of non-selective cation channels. Both passed inward current better than outward (Fig. 4c). Replacement of extracellular Na^+ by Ca^{2+} did not abolish inward current (not shown), indicating that the pore passes Ca^{2+} . Comparison of scaled transient and persistent currents (Fig. 4d) shows that transient current both activates and partially desensitizes within the 20 ms required for solution exchange. Persistent current reaches its peak slowly, but responds with no noticeable lag. The inward-rectification, nonspecific cation permeability, and immediate onset found for both currents are characteristics of ligand-gated P2X receptors^{7–9,23,24}.

Our results support the hypothesis that ATP, acting through P2X3 receptors, activates nociceptors as a consequence of local tissue damage. In particular: (1) physiologically low concentrations of ATP evoke action potentials at neurites of cultured nociceptors; (2) tooth-pulp nociceptors and their nerve endings *in situ* contain P2X3 receptors that probably contribute to both their persistent and transient currents. P2X3 receptors do not mediate ATP-gated currents of stretch receptors. The purpose of these currents in stretch receptors is unclear, but extracellular ATP probably serves non-nociceptive functions in sensory neurons and may mediate mechanosensation²⁷ and neurogenic inflammation²¹; ATP may also act presynaptically on sensory neuron terminals in spinal cord²⁸. Most important, nociceptors and stretch receptors use distinct ATP-gated channels and this may prove valuable in designing new treatments for the selective suppression of pain. □

Methods

Tissue culture. To label nociceptors, maxillary molars of 200–250 g male Sprague–Dawley rats were drilled to the dentin-pulp border and a crystal of DiIc_{18} was placed in each cavity before filling with ZnO dental cement¹⁶. Trigeminal ganglia were dissected and dissociated 7–15 days later. Cells were grown on polylysine/laminin-coated glass or plastic in either F12 medium plus 50 ng ml⁻¹ nerve-growth factor (NGF) at 37 °C in 5% CO_2 , or in L15 medium plus NGF at 23 °C in air; the two culture conditions gave no noticeable difference in ATP responses. For each rat, ~50 cells out of thousands dissociated were labelled. To label muscle-stretch receptors, 5 μl of DiIc_{18} (50 mg ml⁻¹ in dimethyl sulphoxide) was injected into masseter muscle of young rats (50–200 g). The MeN5 was microdissected from brain stem slices 5–15 days later. MeN5 neurons were dissociated and plated on a substrate of glial cells in F12 medium plus NGF at 37 °C in 5% CO_2 . One rat yields 40–100 MeN5 sensory neurons, of which about half are labelled. For electrophysiological examination, both MeN5 and tooth-pulp nociceptors were routinely used 24–48 h after dissociation.

Electrical recording. Action potentials were recorded with a voltage follower amplifier using 5 Mohm pipettes. Pipette solution was (in mM): KCl 55, K_2SO_4 60, MgCl_2 7, HEPES 10, EGTA 16, pH 7.35 with KOH. External solution (mM): NaCl 135, KCl 5, CaCl_2 1, MgCl_2 1, glucose 10, HEPES 10, pH 7.3 with NaOH. For action potential measurements, ATP was applied by pressure from a micropipette.

Whole-cell currents were recorded with a patch-clamp amplifier. Holding potential: -80 mV. Internal solution (mM): KCl 95, NaCl 5, CaCl_2 2, MgCl_2 5, EGTA 10, HEPES 10, pH 7.4 with KOH. External solutions (as above) perfused the vicinity of the cell through 1- μl pipettes with flow controlled by computer-operated solenoid valves. The time course of solution exchange was measured (for example, in Fig. 4d) after each experiment by an osmotically induced change in holding current²⁹.

Immunohistochemistry. Antisera against the carboxy terminus of P2X3 receptor (amino acids 383–397; VEKQSTDSGAYSGH) were generated in New Zealand white rabbits ($n = 4$) and guinea-pigs (Sasco; $n = 4$) as described³⁰. Specificity of the antisera was determined by staining HEK293 cells transfected with P2X3 and by absorption controls with cognate peptide²⁵. Trigeminal ganglia, brainstem and tooth pulp were obtained from 3 male Sprague–Dawley

rats (Harlan; 140–300 g) after perfusion fixation. In two additional rats, infraorbital nerve was exposed after enucleation and crushed at two sites ~2 mm apart, proximal to its entry into infraorbital foramen; after 4 h, animals were fixed by vascular perfusion. Fluorogold (FG) (5% in 0.9% saline; from Fluorochrome Inc.) was injected into the pulp of the first maxillary molar of six additional rats by making a pinpoint exposure at the base of a class I occlusal preparation, which was then sealed with a restorative material to prevent leakage of the dye. Tissues were sectioned at 14 μm in a microtome cryostat, and slide-mounted sections were incubated with antisera³⁰. Primary antisera were diluted 1:500–1,000. Preparations were visualized with lissamine-rhodamine or cyanine 3.18 labelled donkey anti-rabbit or donkey anti-guinea pig IgG used at 1:100–200 by fluorescence microscopy (Olympus BX60F; images d, e in Fig. 3) or by confocal laser microscopy (Bio-Rad MRC-1000 system equipped with a krypton/argon ion laser; images in Fig. 3 a–c, f). To determine the number of trigeminal neurons retrogradely labelled with fluorogold and the degree of colocalization of fluorogold and P2X3 immunoreactivity, FG- and FG/P2X3-positive cells were counted in each section of ganglia and the results corrected for double counting³¹.

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Ligands for ErbB-family receptors encoded by a neuregulin-like gene

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Neuregulins (also called ARIA¹, GGF², heregulin³ or NDF⁴) are a group of polypeptide factors that arise from alternative RNA splicing of a single gene. Through their interaction with the ErbB family of receptors (ErbB2, ErbB3 and ErbB4), neuregulins help to regulate cell growth and differentiation in many tissues⁵⁻⁷. Here we report the cloning of a second neuregulin-like gene, neuregulin-2. The encoded product of the neuregulin-2 gene has a motif structure similar to that of neuregulins and an alternative splicing site in the epidermal growth factor(EGF)-like domain gives rise to two isoforms (α and β). Northern blot and *in situ* hybridization analysis of adult rat tissues indicate that expression of neuregulin-2 is highest in the cerebellum, and the expression pattern is different from that of neuregulins. Recombinant neuregulin-2 β induces the tyrosine-phosphorylation of ErbB2, ErbB3 and ErbB4 in cell lines expressing all of these ErbB-family receptors. However, in cell lines with defined combinations of ErbBs, neuregulin-2 β only activates those with ErbB3 and/or ErbB4, suggesting that signalling by neuregulin-2 is mediated by ErbB3 and/or ErbB4 receptors.

ErbB2, ErbB3 and ErbB4⁸⁻¹⁰ are members of a subfamily of receptor tyrosine kinases that also includes the EGF receptor (EGFR). Signalling through ErbB family receptors is important for regulating cell proliferation and differentiation in many tissues⁵⁻⁷, and deregulation of these signalling pathways is implicated in a variety of cancers¹¹. Although it has been demonstrated that neuregulins can activate ErbB2/3/4 receptors through direct or indirect interaction^{12,13}, additional ligands for ErbB-family receptors may exist¹⁴⁻¹⁶. We used a polymerase chain reaction (PCR) based strategy to search for neuregulin-related sequences in an adult rat cerebellum complementary DNA library and have identified a new neuregulin-like gene¹⁷, neuregulin-2.

Figure 1 shows the deduced amino-acid sequence of neuregulin-2 β , derived from a composite of two overlapping cDNA clones. This composite contains an open reading frame (ORF) encoding a 754-amino-acid protein. Sequence analysis revealed four structural motifs: a putative signal sequence, a C2-type immunoglobulin-like (Ig-like) domain¹⁸, an EGF-like domain (residues 252-297) with its six characteristic cysteines¹⁹, and a putative transmembrane domain (which separates the whole sequence into a 315-residue extracellular domain and a 414-residue cytoplasmic domain). Another neuregulin-2 cDNA clone, with an extra 77-base-pair (bp) exon inserted between the fourth and fifth cysteine residues of the EGF-like domain, encodes an alternatively spliced variant of neuregulin-2 with a different EGF-like domain (see Supplementary Information). This neuregulin-2 isoform also lacks a transmembrane domain, because the insertion of the extra exon causes a frameshift in the downstream sequence and the termination of the ORF 33 amino acids downstream of the EGF-like domain. Neuregulin-2 molecules having two variant EGF-like domains are termed neuregulin-2 α and neuregulin-2 β , respectively. The neuregulin

gene also has a similar alternative splicing site that gives rise to the α - and β -subtypes of neuregulins^{2,3,20}, although neuregulin-2 α and neuregulin-2 β are about equally distant from neuregulin- α or from neuregulin- β . Moreover, there is another alternative splicing site in the cytoplasmic domain of neuregulin-2 in other neuregulin-2 cDNA clones (data not shown), corresponding to the a/b/c-tail splicing site in the neuregulin gene²⁰. Therefore, neuregulin-2 and neuregulin not only have similar sequences, they also have similar gene structures.

A protein-database search revealed that the neuregulin-2 proteins are most similar to neuregulins (and to heregulin- β 1 among the isoforms of the neuregulins). Overall neuregulin-2 β shares 45% identity with heregulin- β 1 (ref. 3) and 40% with GGFII (ref. 2). Apart from the N terminus of neuregulin-2 β , the similarity between neuregulin-2 β and heregulin- β 1 extends through their entire sequence (Fig. 2). On the other hand, the N terminus of neuregulin-2 β has significant identity to that of GGFII (43%) (Fig. 2). The



Figure 1 Deduced amino-acid sequence of rat neuregulin-2 β . Arrowed underline marks the putative signal sequence. The immunoglobulin-like domain is outlined by a dashed box. Solid frame surrounds the EGF-like domain; the six cysteines characteristic of this domain are indicated by asterisks. Potential N-glycosylation sites are marked with arrowheads. The putative transmembrane region is underlined. An arrow points to the potential proteolytic site.

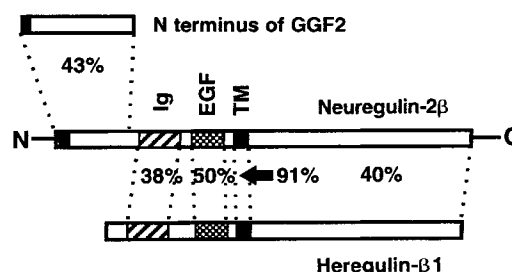


Figure 2 The motif structure of the neuregulin-2 β and its similarity to selected members of neuregulins. The percentage similarity is calculated from the amino-acid sequence alignment of neuregulin-2 β , heregulin- β 1 (human)³ and the N terminus of GGFII (human)². Black boxes, potential signal sequences; Ig, immunoglobulin-like domains; EGF, EGF-like domains; TM, transmembrane domains.

most similar region between neuregulin-2 β and heregulin- β 1 is the transmembrane domain (91% identity) and adjacent sequence. Highly conserved regions also exist in the cytoplasmic tails of neuregulin-2 β and heregulin- β 1, indicating that the cytoplasmic domains may be important biologically. Relatively high conserva-

tion between neuregulin cytoplasmic tails from distant vertebrate species has been noted before¹. As the EGF-like domain of neuregulins is sufficient for receptor binding and for stimulating cellular responses³, we compared the EGF-like domain of neuregulin-2 molecules with other EGF-like motifs. Among the known EGF-like motifs, the EGF-like domain of neuregulin-2 is most similar to that of the neuregulins (48% identity between terminal cysteines in the case of heregulin- β 1). Second to neuregulins is the rat epidermal growth factor, with 43% identity between terminal cysteines.

To determine the size and tissue distribution of neuregulin-2 mRNAs, northern blot hybridization with poly(A)⁺ RNA was carried out using a probe spanning the EGF-like domain plus the immunoglobulin-like domain (Fig. 3A). Among the adult rat tissues examined, neuregulin-2 transcripts were most abundant in neural tissues (brain and spinal cord) and lung. A separate experiment with total RNA samples shows that the cerebellum has the highest concentration of neuregulin-2 transcripts compared to other parts of the brain and other adult tissues (data not shown). Three bands were seen in brain samples (Fig. 3A): a prominent band of 3 kilobases (kb), and two additional bands of 3.8 and 6 kb. Only the 3- and 3.8-kb transcripts were detected in spinal cord and lung samples. This pattern of three principal transcripts has also been found for the neuregulin gene, but at the sensitivity of the northern blot, the tissue distribution of neuregulin-2 transcripts in adult rat seems to be more restricted than that of neuregulins^{3,4}.

We also characterized neuregulin-2 expression by *in situ* hybridization with several probes. In adult rat brain sections, the highest expression was detected in the cerebellum (in the Purkinje cell layer and the granule cell layer) and in the dentate gyrus of the hippocampus (Fig. 3B). Labelled cells were also found in the olfactory bulb (data not shown). This expression pattern seems to be distinct from that of neuregulins, because no hybridization signal for neuregulins is observed in Purkinje cells and very little in the granule cell layer²¹. We investigated the expression pattern of

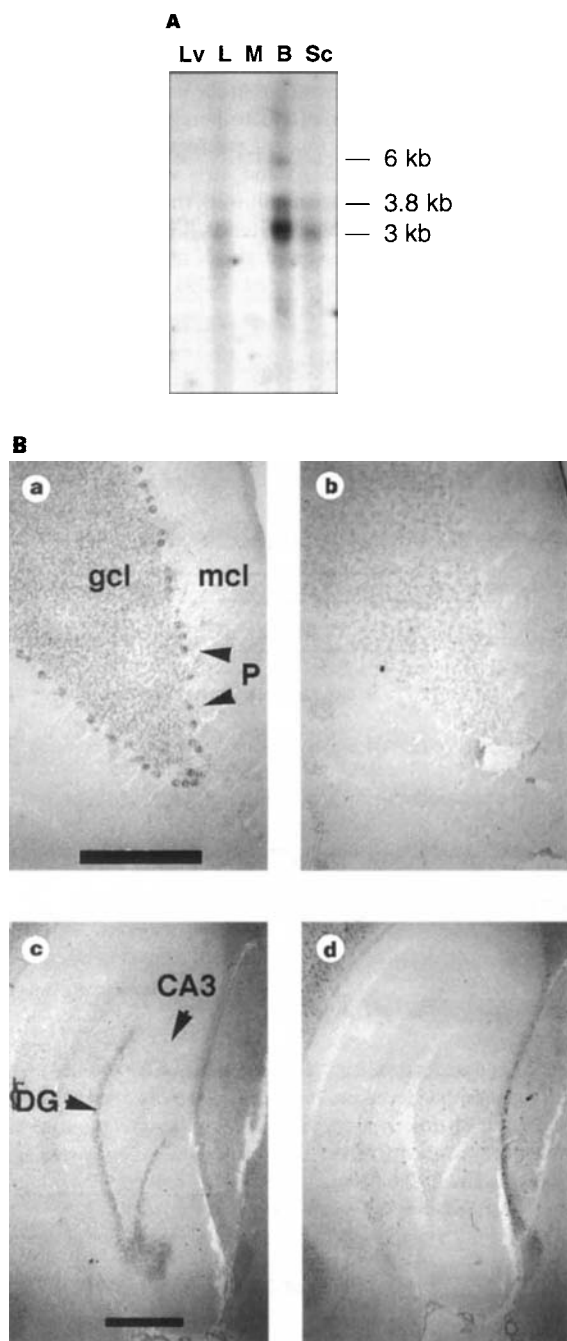


Figure 3 Expression of neuregulin-2 transcripts in adult rat tissues. **A**, Northern blot analysis using poly(A)⁺ RNA samples; approximately 2 μ g poly(A)⁺ RNA was loaded in each lane. The three bands detected (3, 3.8, 6 kb) are indicated. Lv, liver; L, lung; M, skeletal muscle; B, brain; Sc, spinal cord; **B**, *In situ* hybridization of adult rat brain parasagittal sections with a digoxigenin-labelled cRNA probe spanning the EGF-like and Ig domains. **a**, Neuregulin-2 transcripts were detected in Purkinje cells (P) and in the granule cell layer (gcl) in the cerebellum, but not in the molecular cell layer (mcl); scale bar, 0.4 mm. **b**, Adjacent section hybridized with a sense control probe. **c**, In the hippocampus, neuregulin-2 transcripts were only detected in the dentate gyrus (DG) but not in the CA1-CA3 area; scale bar, 0.8 mm. **d**, Adjacent section hybridized with the sense control probe.

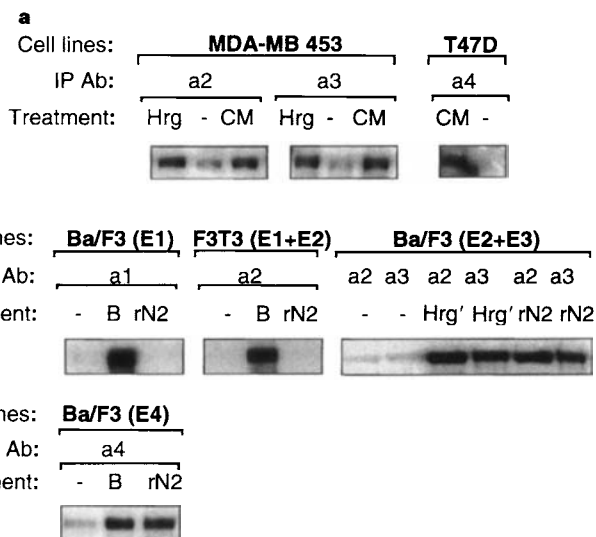


Figure 4 Recombinant neuregulin-2 β protein induces tyrosine-phosphorylation of ErbB-family receptors through ErbB3 and ErbB4. **a**, Neuregulin-2 β induces tyrosine phosphorylation of ErbB2, ErbB3 and ErbB4 in MDA-MB 453 and T47D cell lines. **b**, Neuregulin-2 β signalling through ErbB3 and ErbB4 receptors. rNRG-2 β was tested on cell lines transfected with defined ErbB-family receptors. Only cells with ErbB3 and/or ErbB4 receptors were activated. E1, EGF receptor; E2, ErbB2; E3, ErbB3; E4, ErbB4. Immunoprecipitating antibodies (IPAb): a1, anti-EGF receptor; a2, anti-ErbB2; a3, anti-ErbB3; a4, anti-ErbB4. B, betacellulin; Hrg', heregulin- β 1 EGF-like domain; rN2, rNRG-2 β (EGF-like domain of neuregulin-2 β); CM, neuregulin-2 β -conditioned medium; -, negative control.

neuregulin-2 during embryogenesis by whole-mount *in situ* hybridization with the same probes. We did not detect any signal in E9.5–E10 mouse embryos (data not shown), indicating that none or very little neuregulin-2 is expressed in embryos at those developmental stages. Therefore neuregulin-2 is unlikely to be the 'missing ligand' for ErbB4 receptors¹⁵.

The structural similarity between neuregulin-2 and neuregulins suggests that the neuregulin-2 proteins may also function as ligands for ErbB family receptors. To test this, we expressed a large portion of neuregulin-2 β (including all of the extracellular domain and part of cytoplasmic domain) in CHO cells. As the sequences around the putative proteolysis sites are highly conserved between neuregulin-2 and neuregulins, a soluble form of neuregulin-2 β protein should be released from its precursors to the culture medium, as in the case of neuregulins^{1–4}. We treated cells expressing ErbB family receptors (MDA-MB453 and T47D breast cancer cell lines)^{22,23} with conditioned medium from stably transfected CHO cells. As shown in Fig. 4a, ErbB2, ErbB3 and ErbB4 receptors were activated by neuregulin-2 β -conditioned medium. But as ErbB family receptors can form ligand-induced heterodimers, the activation of ErbB2/3/4 receptors could be due to direct or indirect interaction with neuregulin-2 β . We also expressed the EGF-like domain (amino acids 240–305; Fig. 1) of neuregulin-2 β in bacteria and produced a refolded neuregulin-2 β protein fragment (rNRG-2 β) from inclusion bodies. rNRG-2 β can activate ErbB-family receptors in our breast-tumour cell lines (data not shown), suggesting that like neuregulins, the EGF-like domain is the functional domain for activating ErbB-family receptors.

To determine which of the ErbB family receptors is involved in neuregulin-2 β signalling, we tested rNRG-2 β on cell lines expressing defined combinations of ErbB receptors. We did not detect rNRG-2 β activation of EGF receptors in the Ba/F3 (EGFR) cell line or of ErbB2 receptor in the Fischer rat 3T3 cell line (Fig. 4b), whereas our positive control, betacellulin²⁴, stimulated these receptors. On the other hand, rNRG-2 β stimulated ErbB4 receptor in the Ba/F3 (ErbB4) cell line, as well as ErbB2 and ErbB3 receptors in the Ba/F3 (ErbB2 + ErbB3) cell line. These results indicate that neuregulin-2 β signalling results from direct interaction with ErbB3 and/or ErbB4 receptors.

We have shown that the neuregulin-2 gene, which has structural similarity to the neuregulin gene, encodes new ligands for the ErbB3 and ErbB4 receptors. The distinct expression pattern of neuregulin-2 suggests that these proteins have specific biological functions. It will be necessary to compare neuregulin-2 with neuregulins and other ligands for ErbB-family receptors, including the temporal and spatial regulation of their expression, in order to understand the function of this multiligand/multireceptor signalling network.

Methods

Cloning of neuregulin-2 cDNAs. Two pools of degenerate oligonucleotides were synthesized based on two conserved regions of the neuregulin sequences, one in the immunoglobulin-like domain and the other in the EGF-like domain. Phages from an adult rat cerebellum cDNA library (gift from D. Zhao) were used as templates for PCR. Two steps were used to reduce neuregulin sequences and select neuregulin-related sequences. First, PCR products were digested with *Bcl*I and separated by agarose gel electrophoresis, because there is a *Bcl*I site in rat neuregulin cDNA⁴. DNA fragments of expected sizes were isolated from the agarose gel and reamplified with the same primers. Final PCR products were subcloned into pBlueScriptII vector (Stratagene). Second, individual clones were hybridized with a neuregulin probe under low-stringency conditions and positive clones were sequenced. We identified one clone, n9, which has significant homology to neuregulins. ³²P-labelled probes from the n9 insert were used to screen the cDNA library (~500,000 clones) and several positive clones were identified. The insert of each clone was sequenced in both directions and analysed.

Northern blot and *in situ* hybridization. Poly(A)⁺ RNA was purified from tissues by using a FastTrack kit (Invitrogen). RNA samples were separated on

agarose gels and transferred to nylon filters by standard procedures. Filters were hybridized with ³²P-labelled probes under high-stringency conditions. A probe was generated by random priming of a fragment of neuregulin-2 cDNAs spanning the EGF-like plus the Ig-like domains. The highly conserved transmembrane domain and adjacent sequence were excluded. The probe would hybridize to both neuregulin-2 α and neuregulin-2 β transcripts. *In situ* hybridization was done essentially as described²⁵. We used a digoxigenin-labelled cRNA probe spanning the EGF-like plus the Ig-like domains. Several other probes derived from different parts of neuregulin-2 cDNAs (that is, the EGF-like domain only, the Ig domain only) also gave essentially the same hybridization pattern.

Expression of recombinant neuregulin-2 proteins. The insert of a partial neuregulin-2 β cDNA clone was subcloned into the pRc/CMV expression vector (Invitrogen) and stably transfected into CHO cells. Serum-free conditioned medium was collected. Negative control media were conditioned medium from CHO cells or from CHO cells transfected with an unrelated gene. For expression of rNRG-2 β in *E. coli*, the EGF-like domain of neuregulin-2 β (residues 240–305) was subcloned into pQE32 expression vector with an N terminus 6 $\times$ histidine tag (Qiagen). Protocols for solubilization and refolding of proteins from inclusion bodies were essentially as described²⁴, except that refolded proteins were not purified further. The final rNRG-2 β protein concentration is ~500 μ g ml⁻¹.

Tyrosine-phosphorylation assay. MDA-MB 453 and T47D cells were starved in serum-free medium for 2–6 h before addition of neuregulin-2 β conditioned medium, negative control medium, or heregulin- β 1 (extracellular portion, 20 ng ml⁻¹; from S. J. Burden). After 5–10 min at room temperature, cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1 SDS, 1 mM sodium orthovanadate, 50 μ g ml⁻¹ aprotinin, 0.5 mM PMSF), immunoprecipitated with rabbit antibodies (Santa Cruz Biotechnology) specific for ErbB2 (C18), ErbB3 (C17) or ErbB4 (C18). Immunoprecipitated proteins were collected on protein A-Sepharose beads, analysed by western blotting with an anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology). Antibody binding was detected by enhanced chemiluminescence (Amersham Life Science). The recombinant Ba/F3 cell lines expressing ErbB family receptors have been described, as have protocols for stimulating and analysing ErbB-family receptor tyrosine-phosphorylation^{24,26,27}. EGFR and ErbB2 expression in Fischer rat 3T3 (F3T3) was described²⁸. Human recombinant betacellulin (R&D Systems) was used at 200 ng ml⁻¹. Chemically synthesized heregulin- β 1 65-mer²⁹ was used at 94 ng ml⁻¹. Although we can detect the activity of rNRG-2 β at a dilution of 1:50,000, it was routinely used at a dilution of 1:100 to ensure saturated receptor phosphorylation.

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Neuregulin-2, a new ligand of ErbB3/ErbB4-receptor tyrosine kinases

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The neuregulins (NRGs) are a family of multipotent epidermal-growth-factor-like (EGF-like) factors that arise from splice variants of a single gene. They influence the growth, differentiation, survival and fate of several cell types. We have now discovered a set of new neuregulin-like growth factors, which we call neuregulin-2 (NRG-2): these are encoded by their own gene and exhibit a distinct expression pattern in adult brain and developing heart. Like NRG-1, the EGF-like domain of the new ligands binds to both the ErbB3- and ErbB4-receptor tyrosine kinases. However, NRG-2 stimulates different ErbB-receptor tyrosine-phosphorylation profiles from NRG-1. Our results indicate that NRG-1 and NRG-2 mediate distinct biological processes by acting at different sites in tissues and eliciting different biochemical responses in cells.

The neuregulins (NRG-1) are a family of polypeptide growth factors that are thought to be critical for the developing heart and nervous system, and in the generation and progression of tumours.

In the peripheral nervous system, neuronally derived NRG-1 acts at the neuromuscular junction to promote the end-stage differentiation of muscle cells^{1–4}, and in developing nerves to promote the proliferation, survival and differentiation of Schwann cells^{4–6}. NRG-1 is also essential in the developing heart: neuregulin-deficient mice fail to form ventricular trabeculae and die in mid-gestation^{7–10}. NRG-1 may also play a role in oncogenesis as it can indirectly activate the ErbB2-receptor protein-tyrosine kinase (PTK) whose overexpression correlates with a poor prognosis of some cancer patients¹¹. The molecular cloning of these bioactivities has revealed that NRG-1, separately identified as glial growth factors (GGFs), acetylcholine-receptor-inducing activity (ARIA), heregulins (HRGs), and neu differentiation factor (NDF), are the alternatively spliced products of a single gene^{12–15}. NRG-1 binds to ErbB3 and ErbB4 (refs 16–19), two members of the ErbB subfamily of receptor PTKs, and stimulates the tyrosine phosphorylation of other ErbB receptors through receptor heterodimerization^{19–22}.

We have identified a new NRG-1-like gene called neuregulin (NRG-2). Three distinct complementary DNAs were obtained by screening an adult mouse brain cDNA library with a NRG-1 probe; a composite amino-acid sequence is presented in Fig. 1a. NRG-2 is distantly related to NRG-1 (~35% identity) but exhibits a similar overall domain structure. It includes a single EGF-related motif with the same spacing between the third and fourth cysteine residues as that for EGF (and related molecules that bind to the EGF receptor) but distinct from NRG-1 (see Supplementary Information). NRG-2 carries a single immunoglobulin (Ig)-like domain that is ~36% identical to similar domains found in a subset of NRG-1 splice variants. The transmembrane segment is highly conserved (Fig. 1a, b) and, by analogy with NRG-1, cleavage of the NRG-2 precursor may yield a soluble NRG-2 isoform. The structure of the NRG-2 cDNAs indicates that alternative splicing similar to those found in NRG-1 (refs 12, 14) also generates multiple NRG-2 EGF-like isoforms, including a β 1-like (clone 16A) and an α -like form (clone 5). The splicing alters the carboxy-terminal portion of the EGF-like motif; the NRG-2 and NRG-1 β 1-like forms are particularly highly conserved in this region, with 16/28 identical residues.

To identify sites of NRG-2 expression we analysed, by northern blotting, messenger RNA samples from a series of adult rat tissues (Fig. 2a). Neural expression of NRG-2 mRNA was observed primarily in the cerebellum and olfactory bulb; a transcript was also detected in liver. By whole-mount *in situ* hybridization in mouse E9.5 embryos, NRG-2 mRNA was detected in the endothelial lining of the heart, with the highest mRNA levels being found in the atrium and lower levels in the ventricle and outflow tract (Fig. 2b, c). This profile is complementary to that of NRG-1, which is expressed at high levels in the ventricular endothelium and only weakly in the atrial endothelium⁷. It also differed from NRG-1 in the developing hindbrain, where NRG-1 has been identified in rhombomeres 2, 4 and 6 (ref. 7) and NRG-2 was not detected. In the adult rat, expression of NRG-2 and NRG-1 mRNA was compared by *in situ* hybridization. In the cerebellum, NRG-2 mRNA was detected in granule and Purkinje cells (Fig. 2k), whereas NRG-1 was found mainly in Golgi II cells^{23,24} (Fig. 2j). Cerebellar granule cells also express ErbB4 mRNA (C.L., unpublished results), suggesting a possible autocrine role for NRG-2. In the hindbrain, the NRG-2 mRNA was weakly detectable in the motor trigeminal nucleus (Fig. 2i), compared to a strong NRG-1 signal (Fig. 2h). In the hippocampus, granule cells of the dentate gyrus express NRG-2 (Fig. 2g), where no NRG-1 was detected (Fig. 2f). The cholinergic cells in the basal forebrain that express NRG-1 mRNA^{23,24} (Fig. 2d) are only faintly positive for NRG-2 (Fig. 2e). NRG-2 mRNA was not detected in the hypothalamus^{23,24}, which produces the pituitary-derived NRG-1 known as GGF (not shown). These profiles indicate that in the adult brain NRG-1 and NRG-2 are expressed in largely non-overlapping neural cell populations, suggesting that the two ligands have distinct functional roles.

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The differences in their EGF-like domains suggest that NRG-1 and NRG-2 may have different biochemical properties. As the EGF-like domain of NRG-1 is sufficient for receptor binding and activation and is essential for the activity of the growth factor^{14,16,19}, we expressed these domains as glutathione *S*-transferase (GST) fusion proteins (GST-NRG-1 and -2) and used these for receptor binding and activation. For the binding studies, each of the known ErbB receptors was expressed separately in insect cells and cleared lysates from these cells were mixed with GST, GST-NRG-1 or GST-NRG-2 immobilized on glutathione-Sepharose beads (Fig. 3a). ErbB receptors bound to the beads after washing were visualized by blotting with anti-phosphotyrosine antibodies. Both GST-NRG-1 and GST-NRG-2 bound to ErbB3 and ErbB4 but neither bound to the EGF receptor or to ErbB2.

In three human tumour cells lines that express different sets of ErbB receptors, the two factors elicited different responses, even though they each bind to ErbB3 and ErbB4 (Fig. 3b). Neither factor stimulated a strong response in A549 cells, which express EGF receptor and ErbB2, but both significantly stimulated MDA-MB-453 cells, which express ErbB2, ErbB3 and ErbB4. NRG-1 stimulated weak tyrosine phosphorylation of a 185K protein in MDA-MB-468 cells, which express EGF receptor, ErbB3 and little or no

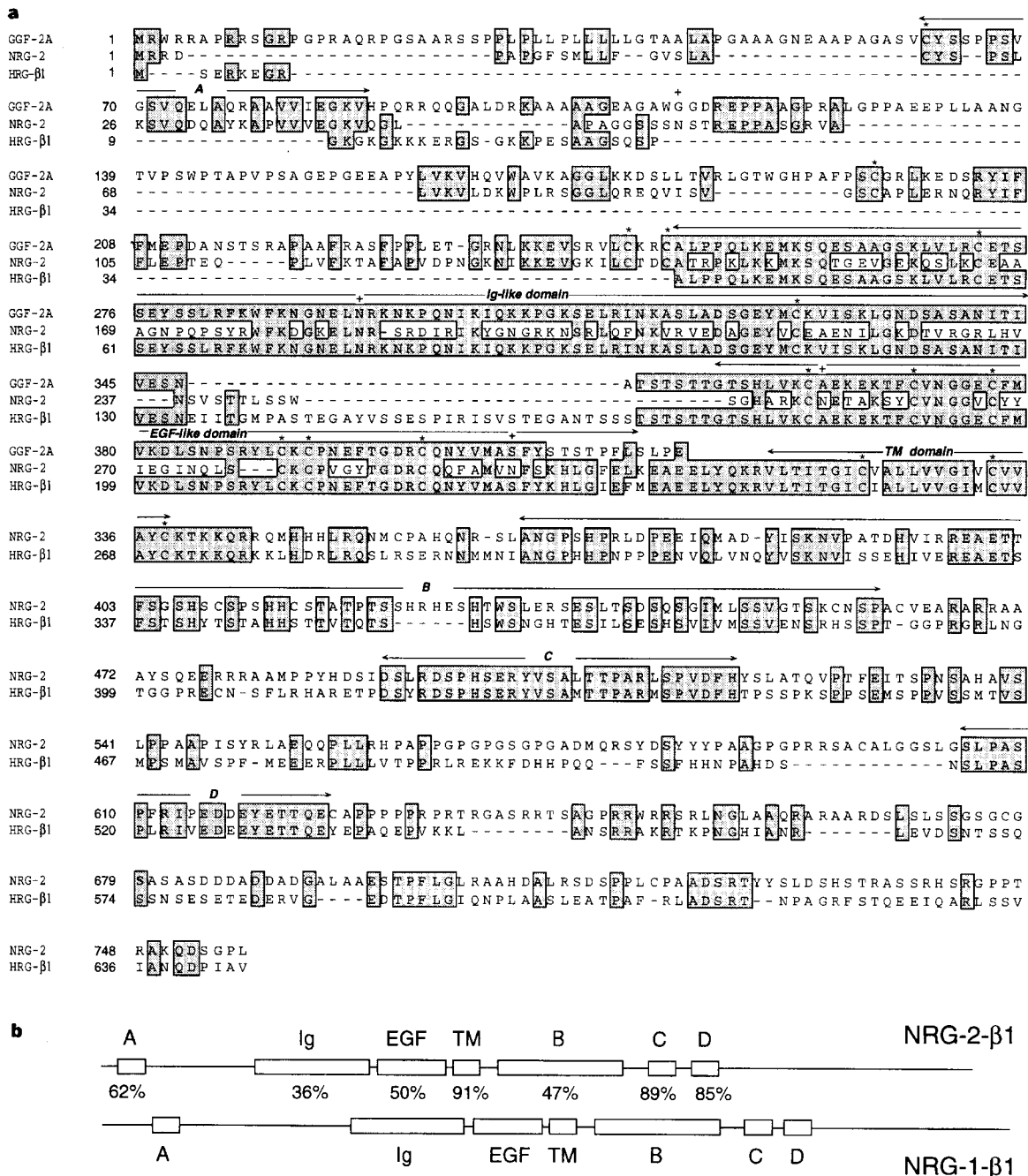


Figure 1 Amino-acid sequence of neuregulin-2 and its variants. **a**, Comparison of NRG-2 amino-acid sequence with human GGF2A (ref. 12) and heregulin-β1 (ref. 14). The assembled sequence contains the entire open reading frame of cDNA clone 16A with the N-terminal 21 amino acids provided by the overlapping cDNA clone 10. Identified domains are delineated. NRG-2 also shares similarity

near the N terminus with GGF2A (block A) and shares three blocks of sequence similarity with heregulin-β1 (blocks B, C, D) in the cytoplasmic tail region. Conserved cysteines are marked with an asterisk. Potential sites of *N*-linked glycosylation in NRG-2 are marked with a plus sign. **b**, Regions of sequence identity between NRG-1 and NRG-2. Blocks A-D are of unknown protein structure.

ErbB4, whereas NRG-2 strongly stimulated phosphorylation of a 175K protein. These observations indicate that the two neuregulins elicit distinct biochemical responses in a cell-type-dependent manner. We compared the ability of the two factors to induce morphological and adhesive changes in MDA-MB-453 cells²⁵. Treatment of these cells with NRG-1 changed 80% of them from a loosely adherent, refractile and spherical appearance to a tightly

adherent, elongated and flattened morphology (Fig. 4a). In contrast, only ~20% responded when treated with NRG-2 under conditions in which these factors fully stimulated tyrosine phosphorylation (Fig. 4b, upper panel). The reduced effect on cell morphology by NRG-2 correlated with a reduction in tyrosine phosphorylation of ErbB2 upon treatment with NRG-2 when compared to NRG-1 (Fig. 4b, upper panel). As stimulation of ErbB2 may be critical for cellular responses to NRG-1 (refs 26,27), the effects of NRG-1 on MDA-MB-453 cells may reflect the stimulation of ErbB2 by this factor. In MDA-MB-468 cells, however, NRG-2 stimulates EGF-R tyrosine phosphorylation more strongly than does NRG-1 (Fig. 4b, lower panel).

The simplest explanation for our observations is that NRG-2 preferentially promotes the heterodimerization of ErbB3 with the EGF receptor, whereas NRG-1 may favour the heterodimerization of ErbB3 or ErbB4 with ErbB2. Such a preference could be related to the structural similarity between NRG-2 and the ligands that bind to the EGF receptor. As each of the ErbB receptors can activate a different set of intracellular signalling pathways by coupling to different Src homology-2 (SH2) domain-containing proteins^{19,28}, the preferential heterodimerization of different receptor pairs by the

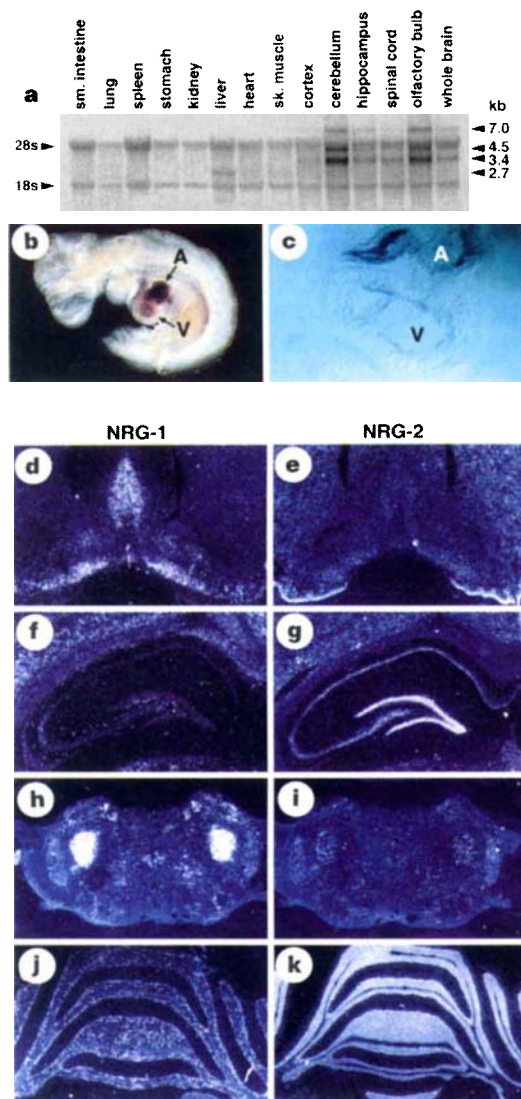


Figure 2 NRG-2 and NRG-1 exhibit distinct patterns of expression in the adult rat brain. **a**, Northern blot showing NRG-2 hybridization to 2 µg poly(A)-selected RNAs prepared from a series of adult rat tissues. NRG-2 is detected in several tissues, with 7.0, 4.5 and 3.4 kb species being observed in brain and a 2.7 kb mRNA in liver. **b**, Expression of NRG-2 in mouse embryos at embryonic day 9.5. NRG-2 mRNA is visualized with digoxigenin-based whole-mount *in situ* hybridization (dark purple reaction product). Staining is prominent in the heart, with a strong signal in the atrium (A) and weaker signals in the ventricle (V) and the outflow tract. Embryo is viewed from its left side. **c**, In a section through the heart of the embryo shown in **b**, NRG-2 mRNA is localized to the endothelial lining of the organ (endocardium) but is absent in the myocardium. **d-k**, *In situ* hybridization of NRG-1- and NRG-2-specific probes to adult rat brain coronal sections. Profiles are shown from septum (**d**, **e**), hippocampus (**f**, **g**), hindbrain (**h**, **i**), and cerebellum (**j**, **k**).

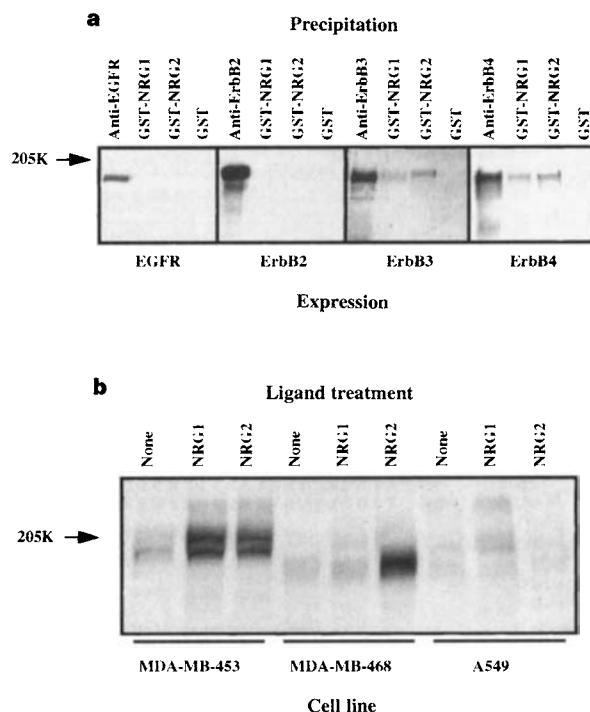


Figure 3 ErbB receptor binding and stimulation of tyrosine phosphorylation by NRG-1 and NRG-2. **a**, Binding of GST-NRG-1 and GST-NRG-2 to insect-cell-expressed receptors. Sf9 insect cells were infected with recombinant baculovirus encoding the indicated receptors, and cleared cell lysates were incubated with glutathione beads containing 3 µg GST-NRG-1, GST-NRG-2 or GST. Insect cells were used because they lack endogenous ErbB receptors. Receptors were also immunoprecipitated with appropriate anti-receptor antibodies as a positive control. Precipitates were immunoblotted with anti-phosphotyrosine to visualize the associated receptors; this is possible because of the endogenous tyrosine phosphorylation of ErbB receptors in insect cells. ErbB3 blots were exposed ~100 times longer than the other receptors because of the very low basal tyrosine phosphorylation. **b**, Stimulation of cancer-cell tyrosine phosphorylation by NRG-1 and NRG-2. MDA-MB-453 and MDA-MB-468 human breast-cancer cells, and A549 human lung-carcinoma cells, were treated for 5 min without growth factor or with 1 µg ml⁻¹ purified GST-NRG-1 or GST-NRG-2, as indicated. Cell lysates were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine. The region of the blot corresponding to the migration of ErbB receptor is shown.

two neuregulins could result in distinct cellular responses. The biological importance of ErbB-receptor heterodimerization is indicated by analysis of transgenic mice lacking either ErbB4 or ErbB2 expression^{8,9}; each of these dies at embryonic day 10.5 from a failure to form ventricular trabeculae in the developing heart. As NRG-1 binds to ErbB4 but not to ErbB2, the phenotype of ErbB2-deficient mice suggests that signalling through an ErbB4/ErbB4 homodimer is not sufficient to rescue the defect and that signalling through an ErbB4/ErbB2 heterodimer may also be required for normal heart development. We conclude that although NRG-1 and NRG-2 have similar receptor-binding specificities, they can stimulate different signalling pathways. As the two factors are expressed in different populations of cells, our findings suggest distinct roles for NRG-1

and NRG-2 in mediating the development of neural and cardiac tissues and in the genesis or progression of tumours. □

Methods

Isolation of neuregulin-2 cDNA clones. NRG-2 cDNA clones were obtained by screening a λZAP mouse (C57B1/6) brain cDNA library at reduced stringency. A NRG-1-specific DNA fragment defined by nucleotide positions 948 to 1,626 of the rat NDF gene¹⁵ was labelled, hybridized to filters containing 4×10^4 PFU each, and washed at a final stringency of $0.2 \times \text{SSC}$, 0.5% SDS at 42°C. Two clone-5 and three clone-10 isolates were obtained. Clone 16A was obtained in a separate screen using an NRG-2 probe.

Northern blotting. RNA and northern blots were prepared as described²⁹. A 1.45-kb *Pst*I/*Sma*I fragment of NRG-2 clone 10 was labelled by random priming. Blots were washed at a final stringency of $0.2 \times \text{SSC}$, 0.2% SDS at 67°C.

In situ hybridization. *In situ* hybridization was done essentially as described²⁹. Slide-mounted paraformaldehyde-fixed sections from adult rat brains were postfixed in 10% formalin before the prehybridization steps. The templates used for probe production were generated using PCR to obtain defined regions of NRG-1 or NRG-2 cDNAs and to introduce the T7 RNA polymerase promoter. For NRG-1, a cDNA clone (C.L., unpublished results) encoding the murine sensory- and motor-neuron-derived factor (SMDF) NRG-1 variant was used to produce a 768-nucleotide SMDF-specific extracellular domain fragment. For NRG-2, a 768-nucleotide insert corresponding to residues 11–267 of the assembled protein sequence (Fig. 1a) was amplified. Transcriptions were done using 250 μCi ³²P-UTP (NEN). Emulsion-dipped slides were exposed for 5 weeks. Slides were counterstained with thionin (except cerebellar tissue).

Whole-mount *in situ* hybridization with digoxigenin-labelled probes and detection with alkaline-phosphatase-conjugated antibody (Boehringer) have been described⁸. After detection, embryos were embedded in 3% agarose, vibratome-sectioned (60 μm), mounted in 90% glycerol/PBS, and photographed under Nomarski optics.

Production of recombinant neuregulin factor and ErbB receptors. Recombinant neuregulin factor was produced in High-Five cells as glutathione S-transferase (GST) fusions with the EGF-like motif of either NRG-1 (β1 form) or NRG-2 (β1-like form). Cassettes containing the EGF-like motifs were generated using PCR and then subcloned into *Bam*HI/*Eco*RI-digested pAcSecG2T (Pharmingen). The secreted proteins have the structure GST–Leu–Val–Pro–Arg–Gly–Ser–[EGF-like motif]. The NRG-1β cassette has the sequence: TSHLIKCAEKEKTFVNGGECFMVKDLSNPSTRYLCKCPNEFTGD RCQNYVMASFYKHLGIEFMEAEELYQK; the NRG-2β cassette has the sequence: SGHARKCNETAKSYCVNGGVCYYIEGINQLSCKCPVGYTGDR CQQFAMVNFSLKHLGFELKEAEELYQK.

Recombinant baculovirus were produced by cotransfection of Sf9 cells with recombinant pAcSecG2T plasmids and BaculoGold viral DNA (Pharmingen). Recombinant proteins were produced by infecting $\sim 2 \times 10^7$ High-Five cells with $\sim 5 \times 10^7$ – 2×10^8 PFU at 27°C for 4 d. The culture medium was harvested, cellular debris were pelleted and the supernatant was mixed with glutathione–Sephadex. The beads were washed with PBS and the fusion proteins eluted with 10 mM glutathione. Samples were stabilized with BSA ($100 \mu\text{g ml}^{-1}$), dialysed into PBS and concentrated. Factor concentrations were estimated by Coomassie-blue staining after SDS–PAGE.

The construction of recombinant baculovirus encoding human EGF-R and bovine ErbB3 has been described³⁰. The construction of recombinant viruses encoding rat ErbB2 and human ErbB4 will be detailed elsewhere (K.L.C., manuscript in preparation). Sf9 (2×10^7) insect cells were infected with recombinant baculoviruses (MOI, 10–30) for 72 h. Cells were resuspended, washed with PBS, lysed in 4 ml lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM orthovanadate, 1 mM ZnCl₂, 5 mM sodium pyrophosphate, 1 mM PMSE, $1 \mu\text{g ml}^{-1}$ each of aprotinin, leupeptin and pepstatin A) and cleared by microcentrifugation. Lysates were split into four aliquots and incubated overnight with glutathione–Sephadex together with either GST (3 μg) or NRG fusion proteins, or were immunoprecipitated with the appropriate antibodies.

Immunoprecipitations. For all immunoprecipitation, insect or cancer cells were lysed in lysis buffer and cleared lysates were immunoprecipitated with

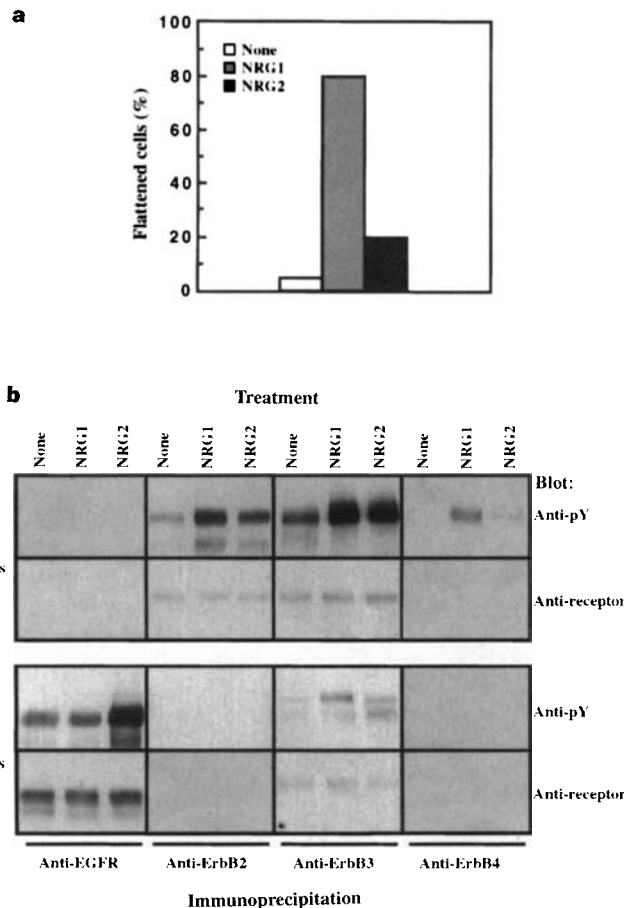


Figure 4 Comparison of the activities of NRG-1 and NRG-2. **a**, Stimulation of breast tumour-cell differentiation by NRG-1 and NRG-2. MDA-MB-453 cells were treated without growth factor or with $1 \mu\text{g ml}^{-1}$ GST-NRG1 or GST-NRG-2 for 96 h, and the percentage of flattened cells determined by microscopy. **b**, Stimulation of the tyrosine phosphorylation of specific ErbB receptors by NRG-1 and NRG-2. MDA-MB-453 (upper panels) and MDA-MB-468 (lower panels) cells were treated without growth factor or with $1 \mu\text{g ml}^{-1}$ purified GST-NRG-1 or GST-NRG-2 for 5 min. Cleared cell lysates were then immunoprecipitated with the indicated anti-receptor antibodies and precipitates were immunoblotted with either anti-phosphotyrosine or the appropriate anti-receptor antibody, as indicated. The region of the blot corresponding to ErbB receptor migration is shown. ErbB4 levels in MDA-MB-453 cells are beneath detection with anti-ErbB4 antibodies.

1 µg of the following anti-receptor antibodies: anti-human EGF receptor 13A9 and anti-ErbB3 3184 (ref. 30), anti-rat ErbB2 Ab-4 (Oncogene Sciences), anti-human ErbB2 3E8, or anti-human ErbB4 Ab-1 (NeoMarkers). 2 µg rabbit anti-mouse was included with the anti-rat ErbB2 and anti-ErbB4 immunoprecipitations to facilitate binding to protein-A-Sepharose. Precipitates were washed three times with lysis buffer, and analysed by 6% SDS-PAGE. Dose-response experiments indicated that the concentration of NRG-2 EGF-like domain required for half-maximal tyrosine phosphorylation is less than 10 nM (K.L.C., unpublished observations). Results were similar when the GST moiety was removed from the NRG-2 EGF-like domain by thrombin proteolysis.

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Ataxia telangiectasia mutant protein activates c-Abl tyrosine kinase in response to ionizing radiation

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Ataxia telangiectasia (AT) is a rare human autosomal recessive disorder with pleiotropic phenotypes, including neuronal degeneration, immune dysfunction, premature ageing and increased cancer risk. The gene mutated in AT, *ATM*, encodes a putative lipid or protein kinase^{1,2}. Most of the human AT patient phenotypes are recapitulated in *Atm*-deficient mice^{3,4}. Cells derived from *Atm*^{-/-} mice, like those from AT patients, exhibit abnormal response to ionizing radiation^{3,5,6}. One of the known responses to ionizing radiation is the activation of a nuclear tyrosine kinase encoded by the *c-abl* proto-oncogene^{7,8}. Ionizing radiation does not activate *c-Abl* in cells from AT patients or in thymocytes or fibroblasts from the *Atm*-deficient mice. Ectopic expression of a functional ATM kinase domain corrects this defect, as it phosphorylates the *c-Abl* tyrosine kinase *in vitro* at Ser 465, leading to the activation of *c-Abl*. A mutant *c-Abl* with Ser 465 changed to Ala 465 is not activated by ionizing radiation or ATM kinase *in vivo*. These findings identify the *c-Abl* tyrosine kinase as a downstream target of phosphorylation and activation by the ATM kinase in the cellular response to ionizing radiation.

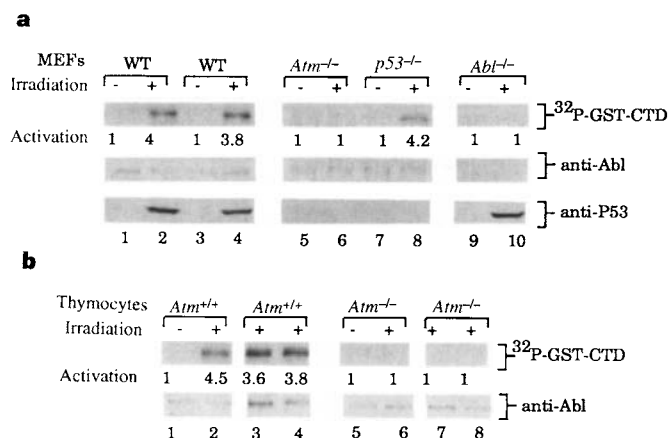


Figure 1 Ionizing radiation does not activate *c-Abl* in *Atm*-deficient cells. **a**, Wild-type (WT), *Atm*^{-/-}, *p53*^{-/-} and *abl*^{-/-} MEFs were collected 1 h after irradiation (10 Gy). The *c-Abl* tyrosine kinase activity was measured using GST-CTD as a substrate. The incorporation of ³²P (top) was corrected for the amount of *c-Abl* in each reaction (middle). Relative increase in activation over unirradiated control was determined. The induction of p53 was also examined (bottom). **b**, *Atm*^{+/+} and *Atm*^{-/-} mice were irradiated (8 Gy) and their thymus collected 1 h later. The *c-Abl* activation in thymocytes was assayed as in **a**.

The ATM function is required for the cellular response to ionizing radiation but not to ultraviolet^{5,9-11}. Similarly, the nuclear c-Abl tyrosine kinase is activated by ionizing radiation but not by ultraviolet⁷. To determine whether there is a functional link between ATM and c-Abl, we examined whether c-Abl could be activated by ionizing radiation in embryo fibroblasts derived from *Atm*-deficient mice. The activity of nuclear c-Abl was measured by a quantitative immune-complex kinase assay using the carboxy-terminal repeated domain (CTD) of RNA polymerase II as a substrate^{7,12}. Activation of c-Abl was observed in two independent wild-type mouse embryonic fibroblasts (MEFs) (Fig. 1a, lanes 1–4). Activity of the kinase was not detected in the *abl*^{-/-} MEFs, demonstrating specificity of the assay (Fig. 1a, lanes 9 and 10). Exposure to ionizing radiation did not activate c-Abl kinase in the *Atm*^{-/-} MEFs, despite the normal level of c-Abl expression (Fig. 1a, lanes 5 and 6). In contrast, activation of c-Abl tyrosine kinase by ionizing radiation was observed in p53-deficient MEFs (Fig. 1a, lanes 7 and 8). In a separate experiment, wild-type or *Atm*-deficient mice were treated with ionizing radiation and the thymus of each was recovered 1 h later. The nuclear c-Abl was extracted from the control and irradiated thymocytes and its activity determined (Fig. 1b). Activation of the thymic c-Abl was observed in three wild-type mice after treatment with ionizing radiation (lanes 1–4). Consistent with the result obtained with the MEFs, activation of c-Abl was not observed in *Atm*-deficient thymocytes (lanes 5–8). The *Atm*-deficient mice were found to develop thymoma at a high rate^{3,4}. Two of the four *Atm*^{-/-} animals used in the experiment contained thymoma, and similar results were obtained with normal and tumour-bearing thymus (Fig. 1b). Taken together, these results show that activation of c-Abl by ionizing radiation is dependent on functional ATM.

Although activation of both c-Abl and p53 by ionizing radiation are dependent on ATM, they are independent of each other. Activation of p53 by ionizing radiation is normal in *abl*^{-/-} MEFs, and the activation of c-Abl is normal in p53-deficient MEFs (Fig. 1a). Thus c-Abl and p53 are distinct downstream targets of ATM.

The product of *Atm* is a large protein with a C-terminal kinase domain homologous to phosphatidylinositol-3-OH kinase, DNA-dependent protein kinase and the yeast checkpoint regulators MEC1, TEL1 and Rad3 (refs 1, 2, 13). Virtually all AT mutations affect the kinase domain, either through frameshifts or point mutations, suggesting that this catalytic function is essential^{11,12,14,15}. Indeed, expression of the kinase domain of human ATM has been shown to rectify the radiosensitivity of cells from AT patients¹⁶. To explore the possible role of ATM kinase in the activation of c-Abl tyrosine kinase, we coexpressed the kinase domain of ATM with c-Abl in *abl*-null 3T3 cells (Fig. 2). The kinase domain of ATM was tagged with the haemagglutinin (HA) epitope, and c-Abl was tagged with the Flag epitope (Fig. 2a). When c-Abl was coexpressed with a functional ATM kinase, its tyrosine kinase activity was increased fourfold (Fig. 2b, lane 3), comparable to the three- to fourfold increase in activation induced by ionizing radiation (Fig. 1). Coexpression with a mutated ATM kinase did not activate c-Abl (Fig. 2b, lane 2). In control experiments, the ATM kinase is shown not to phosphorylate the glutathione-S-transferase (GST)–CTD fusion protein (Fig. 2c, lanes 3 and 5). Thus the ATM kinase domain alone can activate the c-Abl tyrosine kinase in unirradiated mouse cells.

The kinase domain of ATM is homologous to DNA-dependent protein kinase, which preferentially phosphorylates serines followed by a glutamine¹⁷. Such a serine site (DLSQVYE) is present in the c-

Figure 2 ATM kinase activates c-Abl kinase. *Abl*-null 3T3 cells were transfected with the indicated plasmids expressing HA-tagged wild-type (wt) mutant (mut) ATM kinase domain and a FLAG-tagged c-Abl. **a**, Expression of transfected plasmids was verified by immunoblotting with anti-Abl (top) or anti-HA antibody (bottom). **b**, The c-Abl tyrosine kinase was precipitated with anti-FLAG and its activity measured. ³²P-CTD (top) and anti-Abl (bottom) of each reaction is shown and the relative increase in activation determined. **c**, Anti-HA immune complexes were used for CTD phosphorylation (top). The presence of ATM kinase is verified by anti-HA immunoblotting (bottom).

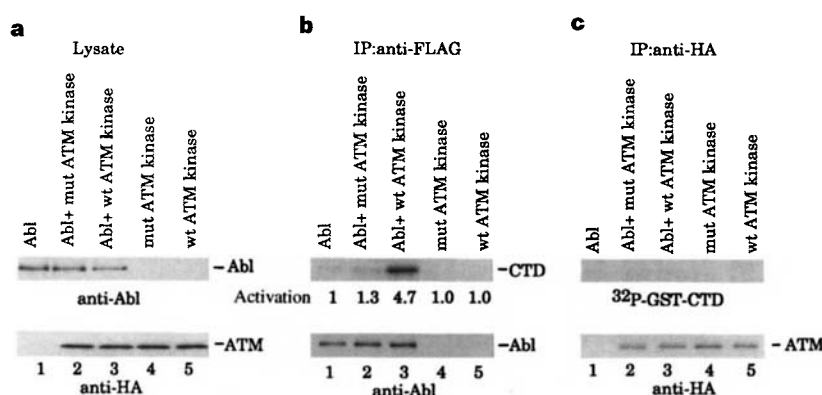
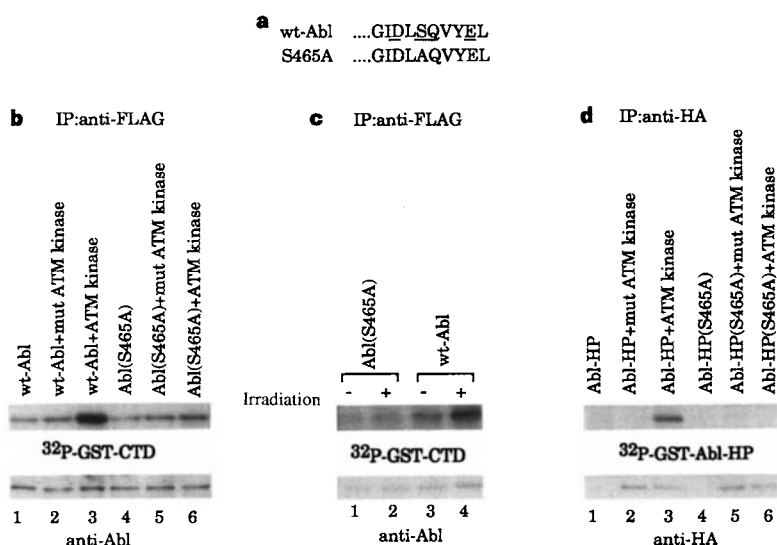


Figure 3 Serine 465 of c-Abl is required for tyrosine kinase activation by ionizing radiation and ATM. **a**, Amino-acid sequence surrounding c-Abl Ser465. **b**, *Abl*-null 3T3 cells were transfected with the indicated plasmids. Wild-type (wt) and S465A mutant (mut) c-Abl was precipitated with anti-FLAG and tyrosine kinase activity assayed. **c**, *Abl*-null 3T3 cells transfected with wt or S465A c-Abl were irradiated (10 Gy). After 1 h, c-Abl was precipitated with anti-FLAG and tyrosine kinase activity measured. **d**, ATM kinase was precipitated with anti-HA from transfected cells, and then incubated with the c-Abl tyrosine kinase domain fragment, purified from bacteria as a GST fusion protein (GST-Abl-HP). ³²P-incorporation into GST-Abl-HP (top) and presence of ATM-kinase (bottom) is shown for each reaction.



Abl tyrosine kinase domain (Fig. 3a). To determine whether this serine is required for activation of Abl by ATM or by ionizing radiation, it was mutated to alanine (Fig. 3a). When cotransfected with the ATM kinase domain, this S465A-Abl mutant did not become activated (Fig. 3b, compare lanes 6 and 3). The S465A-Abl mutant was also refractory to activation by ionizing radiation (Fig. 3c, compare lanes 2 and 4). To test whether ATM kinase can directly phosphorylate c-Abl at this serine, the wild-type and S465A-mutated Abl tyrosine kinase domain was each expressed as a GST fusion protein, purified from bacteria and then incubated with immunoprecipitated ATM kinase (Fig. 3d). Incubation with ATM kinase led to the incorporation of phosphate into the wildtype c-Abl kinase domain fragment (GST-HP) (Fig. 3d, lane 3), but not the S465A mutant fragment (Fig. 3d, lane 6). Incubation with mutant ATM kinase did not lead to c-Abl phosphorylation (Fig. 3d, lane 2). Phosphoamino-acid analysis showed that ATM kinase phosphorylated the GST-Abl-HP fragment on serine (data not shown). Taken together, these results indicate that ATM kinase phosphorylates c-Abl at Ser465 to activate its tyrosine kinase activity.

The ATM-dependent activation of c-Abl by ionizing radiation is also true in human cells (Fig. 4a). c-Abl tyrosine kinase is activated in normal lymphoblasts (Fig. 4a, lane 4) but not in AT lymphoblasts (lane 2), a defect that could be corrected by the expression of the ATM kinase domain. An AT fibroblast line was reconstituted with either vector or ATM kinase domain¹⁶. When exposed to ionizing radiation, c-Abl tyrosine kinase was not activated in the vector-reconstituted AT fibroblasts (Fig. 4a, lane 6), but was activated in the ATM kinase-reconstituted cells (lane 8). The ATM kinase domain can restore the S-phase checkpoint and reduce chromosomal breakage in these AT cells¹⁶. Our results show that the ATM kinase also restores the activation of c-Abl tyrosine kinase induced by ionizing radiation. This is in contrast to the constitutive activation of c-Abl by the coexpressed ATM kinase in transiently transfected *abl*-null mouse 3T3 cells (Fig. 2). That constitutive activation might be due to overproduction associated with transient transfection.

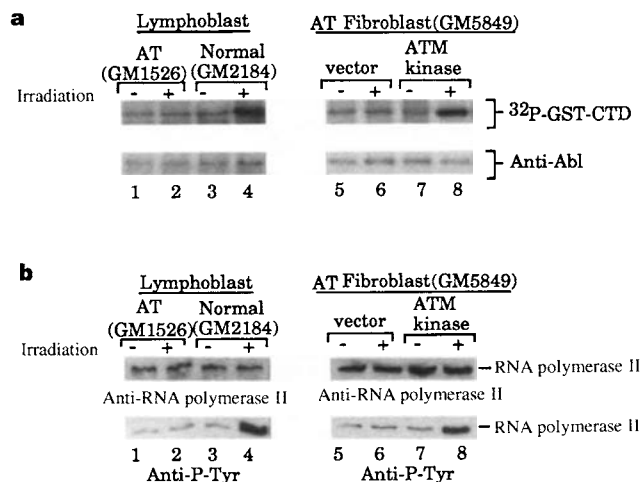


Figure 4 Irradiation-induced tyrosine phosphorylation of RNA polymerase II is dependent on ATM kinase-mediated activation of c-Abl. **a**, An AT fibroblast line (GM5849) was reconstituted with vector or ATM kinase expression plasmid¹⁶. These fibroblasts and lymphoblasts derived from normal (GM2184) or AT (GM1526) individuals were irradiated (8 Gy) and collected 1 h later. The nuclear c-Abl was immunoprecipitated and its tyrosine kinase activity determined. **b**, Nuclear lysates from irradiated (8 Gy) and unirradiated cells were prepared and reacted with anti-RNA polymerase II antibody. The amount of RNA polymerase II in each precipitate determined by immunoblotting with anti-RNA polymerase II (top) and the level of phosphotyrosine determined by immunoblotting with anti-phosphotyrosine antibody (bottom).

Alternatively phosphorylation of c-Abl by the ATM kinase might be regulated more tightly in human cells.

A known substrate for the nuclear c-Abl tyrosine kinase is the CTD of RNA polymerase II^{12,18}. In Abl-positive cells, but not in Abl-null cells, exposure to the DNA-damaging agent MMS leads to the increased tyrosine phosphorylation of RNA polymerase II⁷. We therefore tested whether ionizing radiation also induces tyrosine phosphorylation of RNA polymerase, and whether this is dependent on ATM function. RNA polymerase II is found to contain a low level of phosphotyrosine in unirradiated human cells irrespective of ATM function (Fig. 4b, lanes 1 and 3). Upon exposure to ionizing radiation, the phosphotyrosine level increased in normal lymphoblasts (lane 4) but not in AT lymphoblasts (lane 2). This radiation-induced tyrosine phosphorylation of RNA polymerase II was also observed with ATM kinase-reconstituted AT fibroblasts (lane 8), but

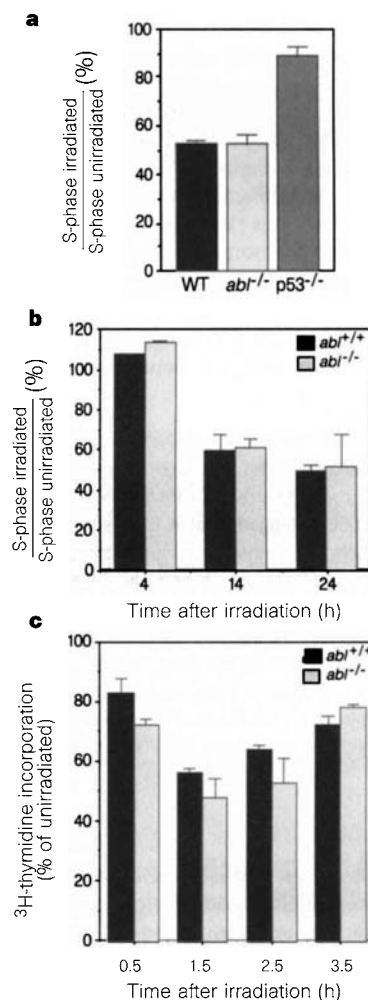


Figure 5 Irradiation-induced cell-cycle checkpoint in *abl*^{-/-} MEFs. **a**, Wild-type (WT), *abl*^{-/-} and *p53*^{-/-} MEFs synchronized in early G1 were irradiated (20 Gy). Cells were collected 23 h later and the proportion of S-phase cells was determined. The percentage of S-phase cells in irradiated culture relative to unirradiated control is shown. Each value represents the mean and standard deviation of two independent experiments. **b**, Exponentially growing MEFs were irradiated and cells were collected 4, 14 or 24 h later and the proportion of S-phase cells determined. Each value represents the mean and standard deviation of three independent experiments. **c**, Exponentially growing MEFs were irradiated and the rate of DNA synthesis determined by pulse-labelling with ³H-thymidine at 0.5, 1.5, 2.5 and 3.5 h after irradiation. The ³H-thymidine incorporation per mg of protein was determined. The value of unirradiated control at each time point was set to 100%. The mean and standard deviation of three independent experiments are shown.

not with the vector-reconstituted control cells (lane 6). These results show that the ATM/c-Abl pathway transduces ionizing radiation signal to RNA polymerase II.

Previous reports have suggested that c-Abl activation might contribute to the cell-cycle arrest induced by ionizing radiation¹⁹. However, we have not observed any checkpoint defects with MEFs derived from *abl*^{-/-} mice. When synchronized cultures were irradiated early in the G₁ phase, S-phase entry was delayed in both *abl*^{+/+} and *abl*^{-/-} MEFs (Fig. 5a). In contrast, S-phase entry was not impeded by ionizing radiation in p53-deficient MEFs (Fig. 5a). With asynchronous MEFs, reduction in S-phase cells was again observed in both *abl*^{+/+} and *abl*^{-/-} cells (Fig. 5b). Thus c-Abl function is dispensable for the G₁/S checkpoint response to ionizing radiation. During S-phase, irradiation causes a transient inhibition of DNA replication. This S-phase checkpoint is defective in AT patient cells²⁰ and in *Atm*-deficient mouse fibroblasts³. The *abl*^{-/-} MEFs, however, showed a similar inhibition in DNA synthesis to the *abl*^{+/+} MEFs (Fig. 5c), demonstrating that c-Abl is not essential for the S-phase checkpoint response to ionizing radiation. We have also examined the radiosensitivity of *abl*^{-/-} and *abl*^{+/+} MEFs by clonogenic assays and found no significant difference (data not shown). We cannot exclude the possibility that c-Abl played a key role in checkpoint response because its function might be replaced by a compensatory mechanism in the *abl*^{-/-} MEFs.

As well as activating cell-cycle checkpoints, ionizing radiation also regulates the transcription of cellular genes^{21–23}. Tyrosine phosphorylation of RNA polymerase II by the c-Abl tyrosine kinase has been correlated with a stimulation of promoter activity¹². Our results suggest that tyrosine phosphorylation of RNA polymerase II mediated by the ATM/c-Abl pathway may be involved in the regulation of RNA polymerase II activity in cells exposed to ionizing radiation. □

Methods

Cell culture, irradiation and transfections. Epstein-Barr virus immortalized lymphoblastoid cell lines from normal individuals (GM 2184) or AT patients (GM1526) were cultured in RPMI supplemented with 15% fetal bovine serum (FBS). SV40-transformed AT fibroblasts (GM5849) reconstituted with vector or human ATM kinase were cultured¹⁶. MEFs were derived from wild-type, *abl*^{-/-}, *p53*^{-/-} and *Atm*^{-/-} embryos, and experiments were done with passage 3 cultures. The γ -irradiation was from a ¹³⁷Cs source emitting a dose of 0.7–7.0 Gy min⁻¹, as calibrated by Freckle dosimetry. Twelve-week-old wild-type or *Atm*^{-/-} mice were irradiated with 8 Gy, using a ¹³⁷Cs source emitting 13 Gy min⁻¹, and were killed 1 h later. Tissues were dissected and snap frozen for use in c-Abl kinase assays. Transient transfections were performed in an Abl-null 3T3 line using lipofectamine (Gibco BRL) according to the manufacturer's protocol. A Flag-tagged Abl in MSCV vector (0.5 μ g) was cotransfected with 2.5 μ g of either a wild-type or a mutated HA-tagged murine ATM kinase domain in pCDNA3.

Plasmids. The Flag-tagged c-Abl was constructed by ligating a 24-base-pair oligonucleotide encoding amino DYLDLDDL into the *Sall* site in the murine *c-abl* coding sequence. The HA-tagged ATM kinase expression plasmid was constructed by PCR as previously described¹⁶. The kinase defective ATM mutant was made by the PCR overlap extension technique, which altered amino acid D2,879 to A, N2,884 to K, and D2,898 to E. The PCR product was subcloned into pCDNA3 (Invitrogen). The ATM kinase constructs were HA-tagged by inserting a 9 amino-acid epitope (YPYDVPDYA) into the *Eco*R1 site at the N terminus of the kinase domain. The S465A mutant of c-Abl was constructed by a two-step PCR using primers that altered the TCT serine codon to a GCT alanine codon. The PCR product was subcloned into the MSCV-Abl-FLAG and GST-Abl-HP²⁴ constructs. The mutation at S465 was confirmed by nucleotide sequencing.

Kinase assay. Immunoprecipitation of the nuclear c-Abl and kinase reaction have been described previously^{7,12}. ³²P incorporation into GST-CTD was determined by phosphorimager and normalized to the amount of c-Abl, determined by anti-Abl immunoblotting, in each reaction. The kinase activity of unirradiated cells was set at 1. For ATM kinase reactions, anti-HA immune

complexes were incubated with GST-Abl-HP in kinase buffer (MOPS, pH 7.2, 25 mM β -glycerophosphate, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride and 1 mM DTT) containing 10 μ M ATP, 20 μ Ci of [γ -³²P]ATP. Reactions were incubated for 30 min at room temperature. The GST-Abl-HP fragments were preincubated with 10 μ M cold ATP before their addition to the ATM kinase reaction.

Antibodies. Immunoprecipitation of c-Abl was performed with the K12 antibody (Santa Cruz), or with the anti-Flag M2 (IBI Kodak) when the Flag-tagged Abl was expressed. c-Abl level was determined by immunoblotting with anti-Abl antibody (8E9). Immunoblotting for p53 was performed using anti-p53 (PAB240; Pharmingen) and immunoblotting for HA-tagged ATM-PI-3 kinase was performed with anti-HA (12CA5, Boehringer Mannheim). Immunoprecipitation of RNA polymerase II large subunit and analysis of its phosphotyrosine content was as described¹².

Cell-cycle analysis and DNA synthesis. To measure the proportion of S-phase cells in asynchronous cultures, cells were pulsed with 10 μ M BrdU for 1 h. To measure the proportion of S-phase cells with synchronous cultures irradiated in early G₁, cells were continuously labelled with BrdU (65 μ M). Cells were fixed in 70% ethanol and stained with a fluorescein isothiocyanate-conjugated anti-BrdU antibody (Pharmingen) and propidium iodide²⁵. The stained cells were analysed with a FACScan (Becton-Dickinson) using the CellFit software. To measure the rate of DNA synthesis, [³H] thymidine was added to a final concentration of 5 μ Ci ml⁻¹ for 30 min. Cells were lysed in 0.1 M NaOH, 10 mM EDTA, 0.5% SDS. TCA-precipitable [³H]thymidine was determined and normalized to the amount of total protein in each lysate.

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Interaction between ATM protein and c-Abl in response to DNA damage

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The gene mutated in the autosomal recessive disorder ataxia telangiectasia (AT), designated *ATM* (for 'AT mutated'), is a member of a family of phosphatidylinositol-3-kinase-like enzymes that are involved in cell-cycle control, meiotic recombination, telomere length monitoring and DNA-damage response¹⁻⁴. Previous results have demonstrated that AT cells are hypersensitive to ionizing radiation⁵⁻⁷ and are defective at the G1/S checkpoint after radiation damage⁸⁻¹⁰. Because cells lacking the protein tyrosine kinase c-Abl are also defective in radiation-induced G1 arrest¹¹, we investigated the possibility that ATM might interact with c-Abl in response to radiation damage. Here we show that ATM binds c-Abl constitutively in control cells but not in AT cells. Our results demonstrate that the SH3 domain of c-Abl interacts with a DPAPNPPHFP motif (residues 1,373-1,382) of ATM. The results also reveal that radiation-induction of c-Abl tyrosine kinase activity is diminished in AT cells. These findings indicate that ATM is involved in the activation of c-Abl by DNA damage and this interaction may in part mediate radiation-induced G1 arrest.

To determine whether c-Abl interacts with ATM, lysates prepared from control and AT lymphoblastoid cells, with or without prior exposure to 5 Gy of ionizing radiation, were subjected to immunoprecipitation with c-Abl antibody. Immunoblot analysis of the anti-c-Abl immunoprecipitate with an anti-ATM peptide antibody (ATM-3BA) demonstrated interaction between these two proteins in the control cell lines but not in the AT cell lines (Fig. 1a). The interaction between c-Abl and ATM was specific because the control lysates precipitated with nonspecific immunoglobulin failed to show an ATM band after immunoblotting with ATM-3BA antibody (Fig. 1a). The association between ATM and c-Abl in control cells is constitutive and is not further induced by exposure to radiation (Fig. 1b). The cell lines used either had mutations that were predicted to give rise to homozygous truncations of the ATM protein (AT3ABR and AT3LA)^{1,12} or a 9-base-pair (bp) in-frame deletion (AT1ABR)¹ capable of producing near full-length protein as determined by immunoblotting (Fig. 1c). Two other AT cell lines

also failed to show interaction (results not shown). The exact mutations for the AT cell lines used are presented in Table 1. Other DNA-damaging agents, including streptonigrin and cisplatin, failed to increase the interaction in control cells or cause the interaction in AT1ABR cells (results not shown).

To determine whether coimmunoprecipitation experiments reflect a direct interaction between ATM and c-Abl, we used the yeast two-hybrid system. Several *ATM* fragments isolated from the full-length complementary DNA were subcloned, in-frame, into pGBT9, pAS2 or pAS2-1 vectors fused to the Gal4 DNA-binding domain (Fig. 2). Full-length *c-Abl* cDNA was cloned into pGAD10 as a fusion to the Gal4 activator domain. All of these constructs fail to activate reporter β -galactosidase expression by themselves. The yeast strain Y190 was cotransformed to express the pair of hybrid proteins and screened for β -galactosidase expression. Full-length c-Abl interacted with a protein coded for by a nearly full-length fragment of ATM, ATM-8.62, residues 3-2,933 (Fig. 2). Two-hybrid interaction analysis revealed that expression of amino-terminal (ATM-3.92, residues 3-1,366) and carboxy-terminal (ATM-4.01, residues, 1,666-3,100) fragments of ATM did not interact with c-Abl, but that a fragment, ATM-4.39 (residues 3-1,466), showed evidence of interaction by filter assay (Fig. 2). This suggests that c-Abl most probably interacts with the portion of the ATM molecule containing residues 1,366-1,466. This region of ATM contains a c-Abl-SH3 consensus binding sequence PXXXXPXX¹³, where c-Abl would be predicted to bind ATM¹⁴. Alternatively, a different binding site may be present in the tertiary structure of ATM-8.62 and ATM-4.39 but absent in ATM-3.92 and ATM 4.01. Although there was evidence of binding between an N-terminal fragment of ATM (residues 3-1,466) and c-Abl, this was lower than that of the nearly full-length ATM cDNA. Quantification of β -galactosidase by liquid culture assay demonstrated an activity of 5.7 ± 0.03 units per 0.5 ml of culture for the nearly full-length ATM cDNA (ATM-8.62) and 2.2 ± 0.04 units for the ATM-4.39 fragment extending from residues 3-1,466. A positive control supplied with the kit (Clontech) gave 80 ± 5 units and the background was 0.02 ± 0.01 units. The results of the yeast two-hybrid analysis suggests a weak interaction between c-Abl and the ATM fragments used in the study. It is possible that the full-length ATM contains tertiary structure that would result in a stronger interaction or that other associated proteins stabilize the association.

In order to confirm this interaction and identify the regions involved in protein binding, *in vitro* binding assays were performed using a GST-Abl-SH3 construct and a larger construct which contains the SH3, SH2 and kinase domains of c-Abl (GST-Abl-SH3/SH2/kin) and *in vitro* translated 5.7-kilobase (kb) partial ATM cDNA¹. The partial ATM cDNA translates as a doublet of about 180K in size. The glutathione-S-transferase (GST) constructs were purified from bacterial lysates by binding to glutathione beads. The beads containing the fusion proteins were incubated with ³⁵S-labelled ATM protein prepared in an *in vitro* coupled transcription/translation reaction. Glutathione beads containing GST fusions were incubated with *in vitro* translated ATM protein in the presence or absence of competing peptide (proline-rich sequence DPAPNPPHFP from ATM), and washed in binding buffer before elution of the bound protein by boiling in SDS. The eluted proteins were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography. GST beads alone and GST-Erk were used as negative controls. ATM was found to bind to both the larger c-Abl construct (Abl-SH3/SH2/Kin), and to the c-Abl-SH3 domain alone. The binding of ATM to GST or GST-Erk was negligible (Fig. 3). Furthermore, the addition of the proline-rich competing peptide, amino acids 1,373-1,382 of ATM, to the GST-c-Abl-SH3 resulted in a marked reduction in the amount of bound ATM at 100-fold excess of peptide (Fig. 3). These results present evidence supporting the binding of ATM to the c-Abl-SH3 domain. The mouse ATM

Table 1 Nature of mutations and ATM protein status in A-T cell lines

Cell line	Mutation	Genotype	ATM protein
AT1ABR	7638 del 9	Homozygote	Nearly full-length
AT3ABR	A 8266 T	Compound heterozygote	Not detectable*
AT5ABR	6412 del AG	Compound heterozygote	Not detectable*
AT3LA	C7491 T	Homozygote	Not detectable*
L3	C103T	Homozygote	Not detectable*

* In all of these cases, mutations were predicted to give rise to truncated proteins^{24,25}.

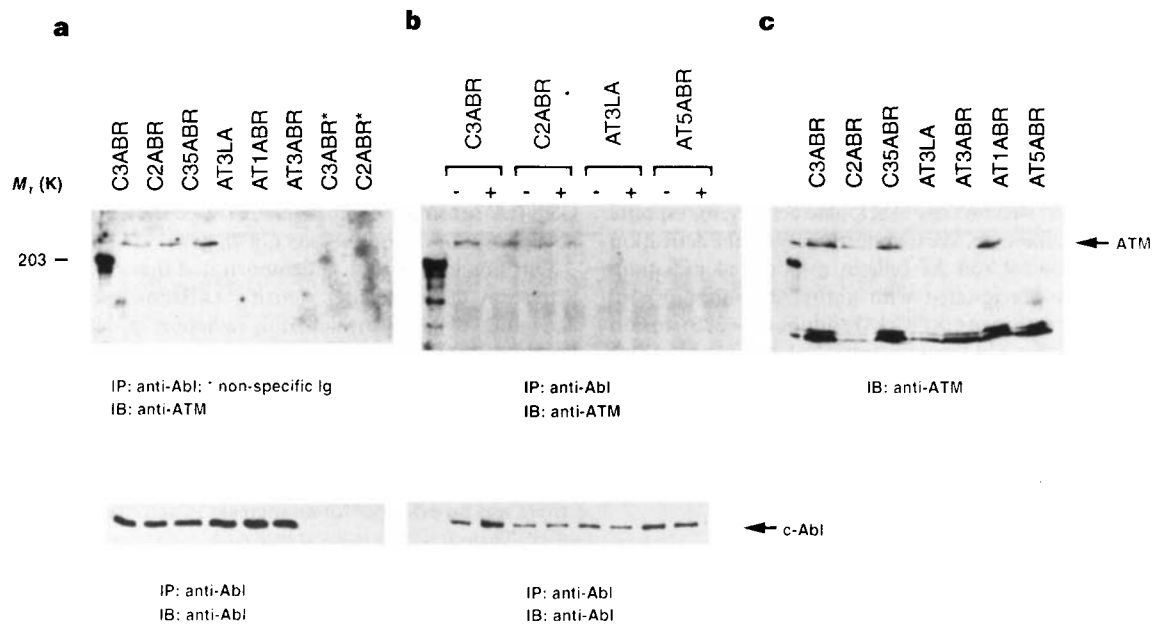


Figure 1 *In vivo* association of ATM with c-Abl. **a**, Total cell lysates were subjected to immunoprecipitation with anti-c-Abl antibody and immunoprecipitated proteins were analysed by immunoblotting with either anti-ATM-3BA antibody (top) or anti-c-Abl antibody (bottom). Precipitation with nonspecific immunoglobulin was included as a control. **b**, Cells were treated with 5 Gy of radiation, incubated for 1 h, lysates were precipitated with anti-c-Abl antibody and bound proteins analysed by immunoblotting with either anti-ATM-3BA antibody (top) or anti-c-Abl antibody (bottom). Plus and minus represent with or without prior

exposure to ionizing radiation. **c**, Western blot showing expression of ATM protein in control and AT cell lines used in the study. C-ABR refers to control lymphoblastoid cell lines and AT-ABR is the corresponding abbreviation for AT cell lines established in Brisbane, Australia²⁰. AT3LA was obtained from R. Gatti (UCLA). L3 is a lymphoblastoid cell line established from a North African Jewish AT patient with a C:T transition at nucleotide 103 of the ORF resulting in a stop codon at position 35 of the ATM protein²⁴.

Figure 2 Summary of the mapping of the c-Abl binding domain in ATM. Different domains of *ATM* cDNA were cloned into yeast shuttle vector plasmids containing Gal4 DNA-binding domain (pGBT9, pAS2, pAS2-1). Full-length *c-Abl* cDNA was cloned adjacent to the Gal4 transactivation domain (pGAD 10). The ATM-8.62 (residues 3–2,933) and ATM-4.39 (residues 3–1,466) constructs were obtained by cloning *Scal*-*Nco*I and *Scal*-*Stu*I *ATM* fragments into pAS2-1, whereas ATM-3.92 (residues 3–1,366) was obtained by cloning *ATM* (*Scal*-*Bam*HI) into pGBT9, and ATM-4.01 (residues 1,666–3,100) by cloning *ATM* (*Nsi*I-*Ase*I) into pAS2. Domains identified in ATM are denoted A, leucine zipper; B, proline-rich sequence; and C, phosphatidylinositol 3-kinase.

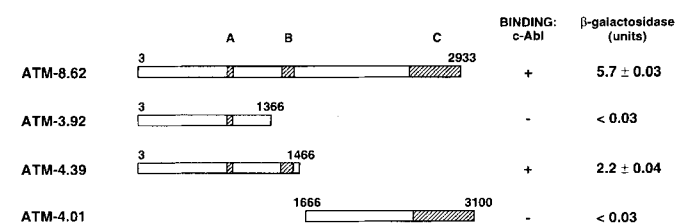


Figure 3 Binding of a proline-rich sequence in ATM to the SH3 domain of c-Abl. *In vitro* translated ATM protein ($0.05 \times$ input radioactivity) was incubated with glutathione agarose beads containing GST alone, GST-Erk, GST-Abl-SH3/SH2/Kin, GST-Abl-SH3 or GST-Abl-SH3 containing 10-fold or 100-fold molar excess of cold peptide (ATM proline-rich sequence, amino acids 1,371–1,384). After binding, beads were washed and the bound proteins were analysed by SDS-PAGE followed by fluorography.



homologue contains an almost identical (His-Tyr substitution at position 8 of the domain) proline-rich sequence in the same relative position in the molecule¹⁵, suggesting a common role in the two species.

Previous studies have shown that the tyrosine kinase activity of c-Abl is activated by DNA-damaging agents, including ionizing radiation^{11,16,17}, and is necessary for radiation-induced G1 arrest¹⁷. However, the upstream effectors of c-Abl kinase activity, in response to DNA damage, are unknown. We therefore analysed the activation of c-Abl kinase in control and AT cells in response to radiation. Lysates were immunoprecipitated with anti-c-Abl antibody and assayed for phosphorylation of GST-Crk (residues 120–225), fusion protein as a measure of c-Abl kinase activity¹⁸. A basal level of Crk phosphorylation was evident in control and AT cells and exposure to ionizing radiation (5 Gy) caused a 2–3-fold increase in Crk kinase activity after 1 h in control cells (Fig. 4a, top). In contrast, there was a minimal increase in c-Abl kinase activity (range 0.6–1.1-fold) in

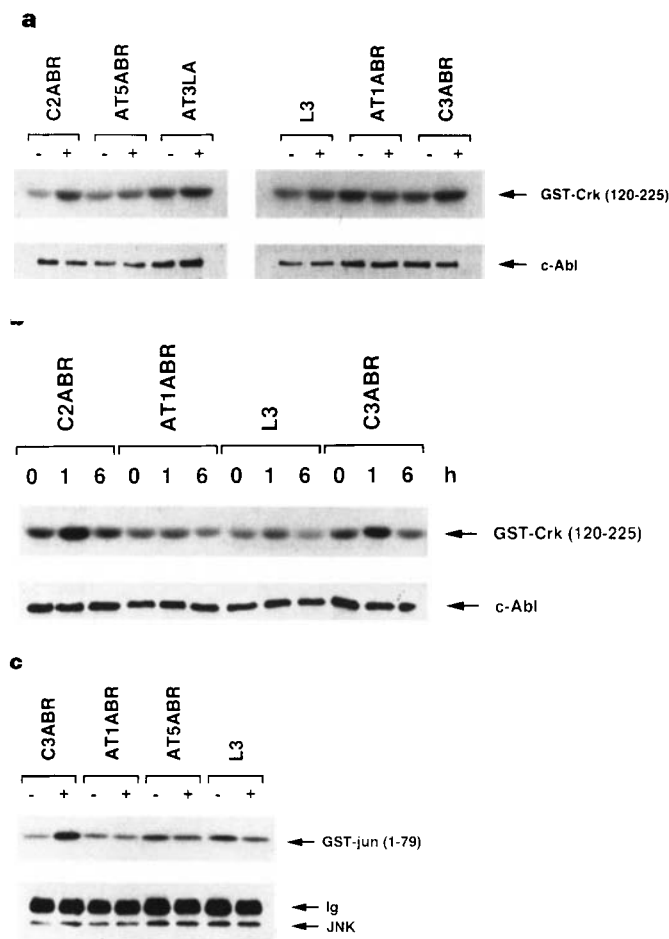


Figure 4 Activation of c-Abl by ionizing radiation. Control and A-T lymphoblastoid cells were treated with 5 Gy of ionizing radiation and incubated for either 1 h (**a**), or 1 h and 6 h (**b**). Total cell extracts were subjected to immunoprecipitation with anti-c-Abl antibody (Ab-3). *In vivo* immune complex kinase assays were performed with GST-Crk (120–225) fusion protein as substrate (top). GST-Crk (120–212) fusion protein (which lacks the critical Tyr221) was used as a negative control. The anti-c-Abl immunoprecipitates were also analysed by immunoblotting with anti-c-Abl antibody (bottom). GST-Crk phosphorylation was quantified by densitometry and normalized according to the amount of immunoblotted c-Abl. **c**, Activation of SAPK by ionizing radiation. Control and AT lymphoblastoid cells were treated with 20 Gy ionizing radiation and collected 1 h later. Total lysates were immunoprecipitated with anti-SAP kinase antibody (JNK1/SAPK β ; Santacruz), and *in vitro* immune complex kinase assays were performed with GST-Jun (residues 1–79) as substrate (top). The anti-SAPK immunoprecipitates were also analysed by immunoblotting with anti-SAPK antibody (bottom).

the four AT cell lines tested. Even when A-T cells were incubated for 6 h postirradiation, there was no evidence of an increase in activity comparable to that observed in controls at 1 h. By 6 h, activity had returned to basal levels in controls (Fig. 4b, top). This change in activity in control cells was not due to changes in the amount of protein because western blot analysis revealed that ionizing radiation had no detectable effect on c-Abl levels (Fig. 4a, b, bottom). A GST-Crk substrate in which Tyr 221 was deleted showed no detectable phosphorylation (results not shown).

Our previous work has demonstrated that c-Abl is required for activation of SAPK after ionizing radiation exposure¹⁷ and that activation of SAPK by ionizing radiation is defective in AT cell lines¹⁹. Accordingly, we assayed for radiation-induced activation of SAPK in the AT cell lines shown here to be defective for c-Abl activation. For SAPK activity, cell lysates were immunoprecipitated with anti-JNK1/SAPK β antibody and assayed for phosphorylation of GST-Jun substrate (residues 1–79). SAPK activity increased 4–5 fold in response to 20 Gy of ionizing radiation in control cells but there was no evidence for an increase in activity in AT cells. (Fig. 4c, top). These findings suggest that ATM is at least in part necessary for activation of c-Abl and SAPK in cells treated with ionizing radiation. The relevance of these results to the defect in the stress response in AT cells is supported by our recent observations, where full-length ATM cDNA is capable of correcting radiosensitivity and re-establishing normal SAPK activation post-irradiation in AT cells²⁰.

Our results suggest that an interaction between ATM and c-Abl is involved in the induction of c-Abl activity in response to DNA damage. AT cells, as well as c-Abl-deficient cells, exhibit defects in p53-dependent radiation-induced G1 arrest^{11,21,22}. Exposure of cells to ionizing radiation also induces c-Abl/p53 complexes and causes a p53-dependent downregulation of Cdk2 and subsequent G1 arrest¹¹. The ATM:c-Abl interaction may thus be involved in the induction of radiation-induced G1 arrest through a signal transduction pathway involving p53. We are currently investigating interactions of ATM and c-Abl with p53 and possible defects in a stress response pathway in AT cells.

Methods

Lysate preparations, coimmunoprecipitation and western blotting.

Control and AT lymphoblastoid cells were irradiated with 5 Gy of radiation (2.8 Gy min⁻¹) and incubated for 1 h. Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 25 mM sodium fluoride, 25 mM β -glycerophosphate, 0.1 mM sodium orthovanadate, 0.1 mM PMSE, 5 μ g ml⁻¹ leupeptin, 1 μ g ml⁻¹ aprotinin, 0.2% Triton X-100, 0.3% Nonidet-P40). After incubation on ice for 30 min, the debris was removed by centrifugation at 14,000g for 15 min, yielding the total cell extract. Anti-c-Abl immunoprecipitations were performed with Ab-3 antibody (Oncogene Science) and protein G-Sepharose overnight at 4°C. Resulting protein complexes were washed three times in lysis buffer and run on 5% SDS-PAGE with an acrylamide:bisacrylamide ratio of 100:1. The separated proteins were transferred in Towbin's buffer at 100 V for 1 h, blocked with skim milk and incubated with ATM-3BA antibody (prepared against an ATM peptide, amino acids 2,581–2,599) for 2 h at room temperature. Antigen-antibody complexes were detected by secondary anti-rabbit antibody followed by enhanced chemiluminescence.

Yeast two-hybrid assay. The protein-protein interactions between ATM and c-Abl were analysed by the yeast two-hybrid method, essentially as per the 'Matchmaker Two Hybrid Kit' from Clontech. The constructs described above were cotransformed into the Y190 yeast strain and transformants were selected on the leu⁻ and trp⁻ media. Positive control and negative control plasmids supplied with the kit were also transformed at the same time. The colonies that appeared after 2–3 days were assayed for β -galactosidase activity by filter assay. Multiple colonies were picked and grown individually in selection media for quantitative β -galactosidase analysis.

In vitro protein interaction. GST-c-Abl-SH3¹⁸ was obtained from D. Baltimore (The Rockefeller University) and the larger c-Abl construct (Abl-SH3/SH2/

Kin) was cloned from cDNA by polymerase chain reaction (PCR) using the forward primer CGGGATCCAGCGGCCAGTAGCATCTGAC and reverse primer CGGAATTCCTTTTCCACTTCGTCGTAG. Underlined sequences indicate the position of *Bam*HI and *Eco*RI sites. The PCR product was digested with *Bam*HI and *Eco*RI and cloned into pGEX-5X-1. GST-Erk 2 (residues 163–199; containing Y 185) was constructed by inserting a *Bgl*II fragment of murine Erk 2 cDNA into the *Bam*HI site of pGEX-3X. GST-abl-SH3 and GST-Erk were expressed in *Escherichia coli* and bound to glutathione-agarose beads²³. These beads (5 µg of GST fusion protein) were incubated with *in vitro* transcribed and translated ATM protein in binding buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% NP40) for 1 h at 4 °C. A 5.7-kb partial ATM cDNA as described previously¹ was used as a template in an *in vitro* coupled transcription/translation reaction (Promega) to produce ³⁵S-labelled ATM protein. The sequence specifying the proline-rich region is present in this cDNA. In two reactions either 10-fold or 100-fold molar excess of ATM peptide (proline-rich region of ATM, residues 1,371–1,384) was added to compete against the binding. Following binding, beads were washed and bound proteins were analysed by SDS-PAGE, followed by fluorography.

Kinase assays. Activation of c-Abl was determined with murine c-Crk as a substrate and GST-Jun (residues 1–79) was used as a substrate to measure activation of SAPK. The murine c-Crk (residues 120–225) and c-Crk (residues 120–212; lacking Try 221) were cloned from cDNA by PCR using the 5' primer Crk-120 CGTGGATCCAGATCAAGGCAGGGTAGTGG and the 3' primers Crk-225 (wild type) CGATGAATTCCTGGCTGTGGGTGGGAACCTCCTGG and Crk-212 (lacking Tyr 221) CGATGAATTCCTGGGTGGGCATAGGGCCCAGG. Underlined sequences indicate the position of *Bam*HI and *Eco*RI sites. The PCR products were digested with *Bam*HI and *Eco*RI and cloned into pGEX-2T. Expressing clones were selected and used in c-Abl activity assays.

For c-Abl activation, control and AT cells were treated with 5 Gy (2.8 Gy min⁻¹) of ionizing radiation. Total lysate was prepared as described above. Anti-c-Abl immunoprecipitations were performed by adding the Ab-3 antibody (Oncogene Science) and protein G-Sepharose for 2 h at 4 °C. Immune complex kinase assays were performed by incubating the resulting protein complexes in kinase buffer (25 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 1 mM MnCl₂, 0.5 mM DTT, 10 µM ATP) with either 5 µg of GST-Crk (120–225) or GST-Crk (120–212), 5 µCi [γ-³²P]ATP for 30 min at 28 °C and analysed by 12% SDS-PAGE and autoradiography. For SAPK activation, control and AT cells were treated with 20 Gy of ionizing radiation. GST-Jun fusion protein was prepared as described previously²³ and immune complex kinase reactions were performed as described above.

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The dynamics of a pre-mRNA splicing factor in living cells

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Pre-mRNA splicing is a predominantly co-transcriptional event which involves a large number of essential splicing factors^{1,2}. Within the mammalian cell nucleus, most splicing factors are concentrated in 20–40 distinct domains called speckles³. The function of speckles and the organization of cellular transcription and pre-mRNA splicing *in vivo* are not well understood. We have investigated the dynamic properties of splicing factors in nuclei of living cells. Here we show that speckles are highly dynamic structures that respond specifically to activation of nearby genes. These dynamic events are dependent on RNA polymerase II transcription, and are sensitive to inhibitors of protein kinases and Ser/Thr phosphatases. When single genes are transcriptionally activated in living cells, splicing factors leave speckles in peripheral extensions and accumulate at the new sites of transcription. We conclude that one function of speckles is to supply splicing factors to active genes. Our observations demonstrate that the interphase nucleus is far more dynamic in nature than previously assumed.

In an attempt to study the dynamic aspects of nuclear organization, we sought to visualize pre-mRNA splicing factors in living cells. The green fluorescent protein (GFP)⁴ was fused in-frame to the amino terminus of the essential splicing factor SF2/ASF^{5,6}. When expressed in several cell lines, the GFP-SF2/ASF fusion was indistinguishable from endogenous pre-mRNA splicing factors, as assessed by four criteria (Fig. 1). First, both endogenous SF2/ASF and GFP-SF2/ASF were phosphorylated *in vivo*, because they migrated as multiple bands of relative molecular mass (*M_r*) 30K to 35K, and 60K to 70K in an SDS-polyacrylamide gel (Fig. 1A, lanes 1 and 3), respectively, and shifted into single bands of 30K and 57K, respectively, after alkaline phosphatase treatment (Fig. 1A, lanes 2 and 4). Second, GFP-SF2/ASF localized correctly to the nucleus (Fig. 1B, a), where it colocalized in morphologically normal speckles with the endogenous pre-mRNA splicing factor U2-B'' (Fig. 1B, b) and splicing factors containing the Sm epitope. GFP-SF2/ASF and all endogenous splicing factors were also present in a diffuse nucleoplasmic pool (Fig. 1B). Third, GFP-SF2/ASF was functionally active *in vivo*. In a β-thalassaemia reporter (Fig. 1C, bottom), the decision as to which of three available cryptic 5' splice sites is used *in vivo* is determined by the cellular ratio of

functional SF2/ASF to the hnRNP protein A1 (ref. 7). In the presence of endogenous levels of SF2/ASF, cryptic splice site 2 is used predominantly (Fig. 1C, lane 1). A switch to cryptic splice site 3 occurs when the SF2/ASF concentration is increased by expression of functional SF2/ASF⁷ (Fig. 1C, lane 2). Expression of GFP-SF2/ASF, like SF2/ASF, caused a switch to cryptic splice site 3 (Fig. 1C, lane 3), demonstrating that GFP-SF2/ASF functions correctly in selection of alternative splice sites *in vivo*; expression of GFP alone did not induce this switch (Fig. 1C, lane 4). Fourth GFP-SF2/ASF accumulated at sites of active transcription (Fig. 1D). When intron-containing genes are transiently expressed, pre-mRNA splicing factors accumulate at sites of plasmid transcription^{8,9}, presumably because they are involved in the co-transcriptional excision of introns. When an intron-containing β -tropomyosin minigene¹⁰ was transiently expressed in baby hamster kidney (BHK) cells, GFP-SF2/ASF (Fig. 1D, b) accumulated at sites of plasmid transcription (Fig. 1D, a), as expected for a functional splicing factor.

To determine the degree to which nuclear speckles are mobile within the cell nucleus of living BHK cells expressing GFP-SF2/ASF,

we used time-lapse fluorescence microscopy (Fig. 2; see Methods). During observation periods, ranging from 45 min to 8 h, most speckles (>80%) remained stationary with respect to their overall position within the nucleus (Fig. 2a, arrows). Less than 20% of speckles fused (arrowhead in Fig. 2a) or budded from each other, and a few disappeared or formed at new sites. Although the position of most speckles within the nucleus was generally unaltered, distinct changes in the shape of virtually all speckles were observed. To assess these movements, images of single cells were taken over 45 min at intervals of 1.5 min and analysed in video format (see Supplementary Information). Selected frames in pseudocolours (speckles appear red) are shown in Fig. 2b,c. Two types of dynamic events were clearly distinguished: extensions ranging in length from 300 nm to more than 1 μ m formed regularly from the periphery of speckles and typically persisted for an average of 5–7 min (Fig. 2b) and what seemed to be small particles frequently associated with and dissociated from speckles (Fig. 2c). Both types of movements were at times observed simultaneously in the same speckle. The movements were highly reproducible, and were not caused by

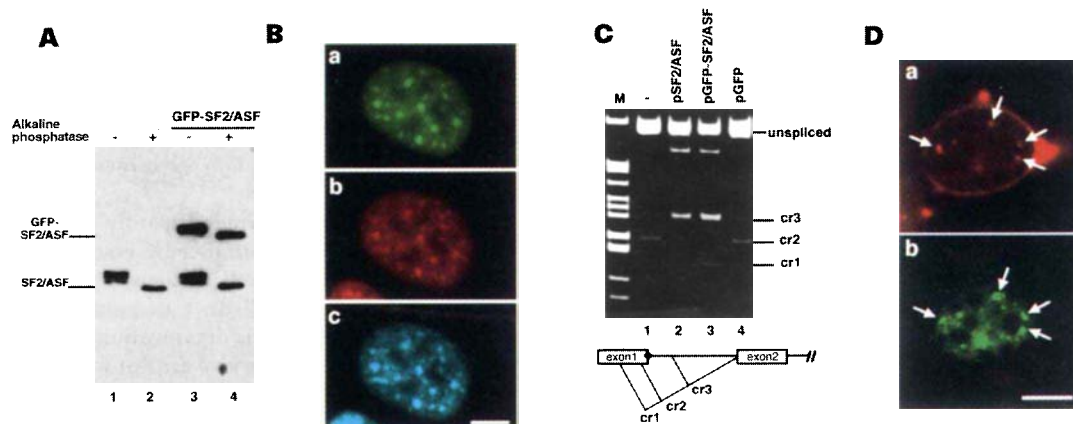
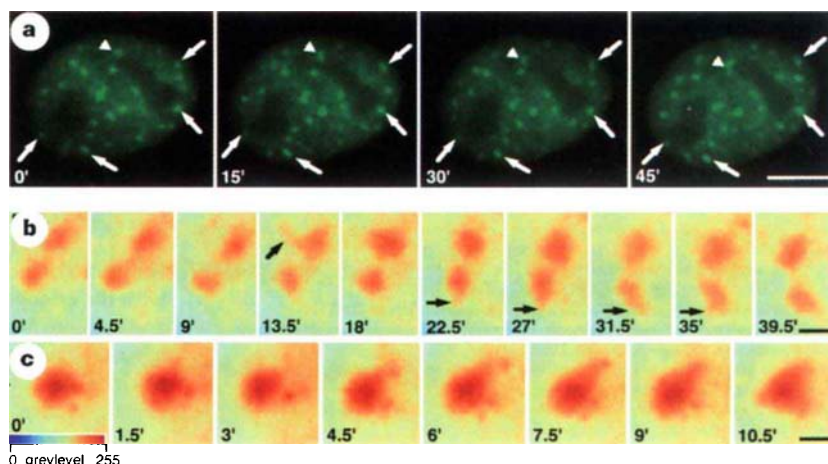


Figure 1 Characterization of the GFP-SF2/ASF fusion protein. **A**, Immunoblot analysis of GFP-SF2/ASF expression 14 h post-transfection using anti-SF2/ASF antibody. Lanes 1 and 2, control cells; lanes 3 and 4, transfected cells; lanes 2 and 4, alkaline phosphatase-treated lysates. Both endogenous SF2/ASF and GFP-SF2/ASF were phosphorylated *in vivo*. **B**, GFP-SF2/ASF colocalized with endogenous pre-mRNA splicing factors in nuclear speckles. GFP-SF2/ASF (**a**) localized exclusively to the nucleus and colocalized with splicing factor U2-B'' (**b**; anti-mouse-Texas red secondary antibody) and several splicing factors containing the Sm epitope (**c**; anti-human-Cy5 secondary antibody). Scale bar, 5 μ m. **C**, GFP-SF2/ASF was active in alternative splice-site selection *in vivo*. HeLa cells were cotransfected with a β -thalassaemia reporter substrate (bottom) and the

indicated SF2/ASF construct. Splice site selection was analysed as described⁷. In control cells, cryptic splice site 2 is used predominantly (lane 1). Upon expression of either SF2/ASF (lane 2) or GFP-SF2/ASF (lane 3), a switch to the most proximal 5' splice site 3 occurred. GFP alone did not cause this shift (lane 4). M, electrophoresis marker. **D**, GFP-SF2/ASF accumulated at the site of transcription of exogenous transcripts. BHK cells were cotransfected with GFP-SF2/ASF and β -tropomyosin minigene, cells were fixed at 6 h post-transfection; β -tropomyosin RNA was detected by fluorescence *in situ* hybridization; β -tropomyosin RNA (**a**) and GFP-SF2/ASF (**b**) colocalized at sites of plasmid transcription (arrows). Scale bar, 7 μ m.

Figure 2 Time-lapse fluorescence microscopy of GFP-SF2/ASF in living BHK cells. Images were taken at 1.5-min intervals and the time is indicated in minutes in the bottom left-hand corner of the image. **a**, The position of most speckles within the nucleus did not change significantly over time (arrows). Some speckles fused (arrowhead) or changed their nuclear position. **b**, Distinct changes in shape and peripheral extensions (arrows) were observed in virtually all speckles. **c**, What appeared to be particles were frequently seen to dissociate from or associate with speckles. The spectrum of pseudocolours represents the intensities of the fluorescence signal measured for each pixel (blue, lowest; red, brightest). Speckles appear in red against a green background representing the nucleoplasmic pool of GFP-SF2/ASF. Scale bars: **a**, 6.5 μ m; **b**, 750 nm; **c**, 500 nm.



movement of the cell itself, as cells with varying degrees of cell mobility exhibited similar speckle dynamics. Identical observations were made in HeLa, Swiss 3T3 and Detroit 551 cells, and with constructs encoding amino- or carboxy-terminal fusions of GFP to either splicing factor SF2/ASF or SC35 (ref. 11) and driven by a variety of promoters. No peripheral movements were seen in fixed cells, excluding the possibility that the movements were caused by focal drift (data not shown).

As splicing of most pre-mRNAs occurs co-transcriptionally¹, we tested how the dynamic events observed related to RNA polymerase II transcription. Peripheral movements were strictly dependent on ongoing RNA polymerase II transcription (Fig. 3a). In cells imaged after incubation for 2 h with 50 $\mu\text{g ml}^{-1}$ α -amanitin, a specific inhibitor of RNA polymerase II (ref. 12), neither peripheral extensions nor (dis)associating particles were observed (Fig. 3a). Less than 2% of speckles displayed detectable peripheral movements. This was in contrast to 82% of speckles which showed significant peripheral movements in the presence of ongoing transcription.

Protein (de)phosphorylation has been implicated in the nuclear distribution of splicing factors^{13–15}. To test whether protein phosphorylation also had an effect on the dynamic behaviour of GFP-SF2/ASF, transfected BHK cells were incubated either with an inhibitor of protein kinases (staurosporine)¹⁶ or Ser/Thr protein phosphatases 2A and 1 (okadaic acid)¹⁷; the latter phosphatase dephosphorylates SR proteins *in vitro* and in permeabilized cells^{18,19}. Neither drug had any aberrant effect on overall nuclear morphology under the conditions used (Fig. 3b,c), although staurosporine caused a slight rounding up of some speckles (Fig. 3b). However, staurosporine blocked all dynamic movements at the periphery of speckles (Fig. 3d). The opposite was seen in the presence of okadaic acid, which caused speckles to become less well defined at their periphery, and the pool of GFP-SF2/ASF in the nucleoplasmic space seemed to increase in amount (Fig. 3c,e). These data are consistent with reports that phosphorylation of SR-protein splicing

factors results in their release from speckles^{13,14}.

The ability to observe the dynamic movements of splicing factors allowed us to visualize how pre-mRNA splicing factors respond to the transcriptional activation of nearby genes in the nucleus of a living cell. BKT-1B cells, derived from BHK cells, carry an integrated full-length BK-virus genome, and expression of BK virus early genes can be triggered within 60 min by cyclic AMP²⁰. The speckled pattern of BKT-1B cells transfected with GFP-SF2/ASF was recorded at 14 h post-transfection, BK gene transcription was then triggered, and the behaviour of nuclear speckles was followed by time-lapse microscopy (Fig. 4a). Within 15–20 min of gene activation, a trail of splicing factors formed gradually from one or occasionally two neighbouring speckles (Fig. 4a). The trail extended in length and increased in signal intensity over the next 30 min (Fig. 4a). The redistribution was directed towards the new site of transcription, which was visualized by fluorescence *in situ* hybridization using probes specific for the intron-containing BKV-virus RNA (Fig. 4a). Identical observations were made in rat R9 cells, which carry a cycloheximide-inducible copy of the cytomegalovirus (CMV) immediate early genes^{21,22} (Fig. 4b). In the cell shown in Fig. 4b, peripheral extensions formed gradually from two neighbouring speckles (arrowheads), and were fully prominent at 4 h (Fig. 4b), the time required for maximum expression of the intron-containing CMV-IE genes²¹. Again, the position of the newly formed speckle extension coincided with the newly induced site of transcription (Fig. 4b). These observations demonstrate that nuclear speckles supply pre-mRNA splicing factors to nearby activated sites of transcription.

Visualization of nuclear components in living cells has allowed us to characterize dynamic events within the interphase cell nucleus. We have shown that pre-mRNA splicing factors are dynamic within the interphase nucleus and are rapidly recruited from speckles to sites of transcription after gene activation. Our observations directly demonstrate that one function of speckles is to supply pre-mRNA

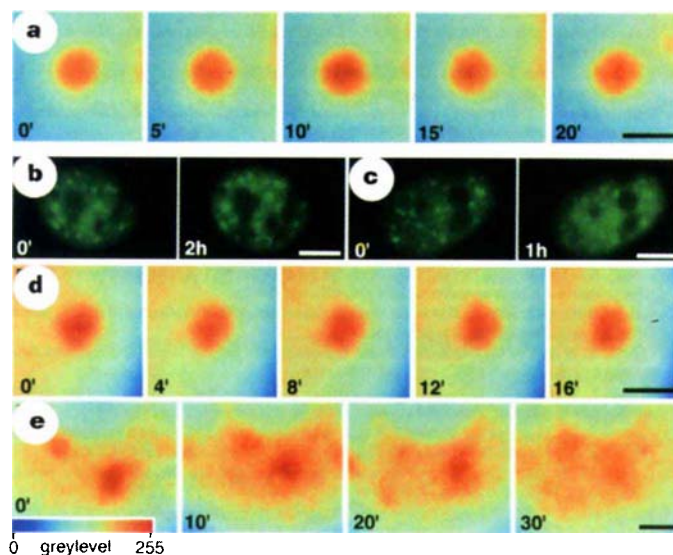


Figure 3 Speckle movements are sensitive to inhibitors of RNA polymerase II (α -amanitin), protein kinases (staurosporine), and Ser/Thr phosphatases 1 and 2A (okadaic acid). **a**, Addition of 50 $\mu\text{g ml}^{-1}$ α -amanitin for 2 h caused a marked rounding up of speckles and completely prevented peripheral movement. **b**, **c**, Staurosporine for 2 h (**b**) or okadaic acid for 1 h (**c**) had no significant effect on overall nuclear morphology. **d**, Staurosporine prevented all dynamic, peripheral movements. **e**, Okadaic acid caused a gradual increase in the nucleoplasmic pool of GFP-SF2/ASF. Images were taken at the indicated timepoints after 60 min (**d**) or 15 min (**e**) of incubation. Images are pseudocoloured; speckles appear in red. Times (min) are given in the bottom left-hand corner. Scale bars: **a**, **d**, 500 nm; **b**, **c** 5 μm ; **e**, 720 nm.

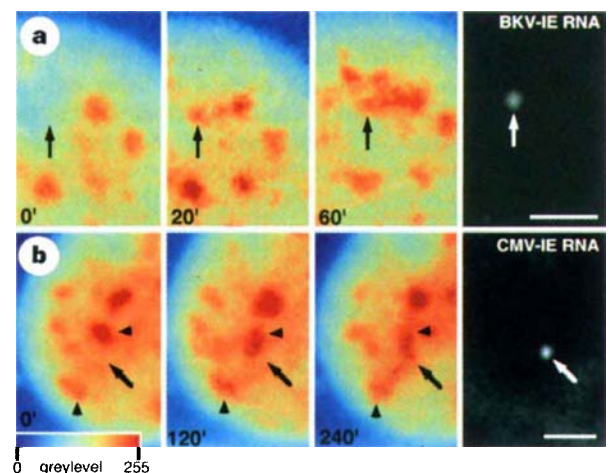


Figure 4 GFP-SF2/ASF responds to transcriptional activation of genes by redistribution to sites of transcription. Transcription of BK-virus (**a**) or CMV-IE genes (**b**) were triggered by addition of 50 $\mu\text{g ml}^{-1}$ cAMP or 50 $\mu\text{g ml}^{-1}$ cycloheximide, respectively. Images of speckles after induction of transcription were taken at the indicated time points. After acquisition of the final image, the cell was fixed and the position of the induced RNA detected by fluorescence *in situ* hybridization. Pre-mRNA splicing factors formed trails from existing speckles in the direction of the induced genes. Note that two neighbouring speckles (arrowheads in **b**) respond to gene activation by forming peripheral extensions. Images are pseudocoloured; speckles appear in red. Times (min) are given in the bottom left-hand corner. Arrow, position of the RNA signal. Scale bars: 1.5 μm .

splicing factors to sites of active transcription, as previously proposed^{8,9,22,23}. This interpretation is supported by observations using deconvolution microscopy on fixed cells that showed that transcriptionally active genes, and their nascent RNAs in many cases are located at the periphery of speckles²⁴. Further, electron microscopic studies show that transcription occurs in nuclear structures called perichromatin fibrils²⁵, which frequently localize to the periphery of speckles, and also in nuclear regions away from speckles. Recruitment of splicing factors to sites of transcription is also supported by biochemical evidence indicating that splicing factors physically interact with the C-terminal domain of RNA polymerase II (refs 26, 27). The physical association of certain 3' processing factors with RNA polymerase II has recently been reported, and it is possible that other nuclear events, such as polyadenylation, are similarly coordinated with transcription²⁸.

Our data do not exclude the possibility that activated genes also move towards an existing speckle. In addition, it is not clear whether splicing factors are supplied exclusively by speckles or whether some factors are also recruited from the nucleoplasmic pool. However, the formation of trails extending from existing speckles towards the induced sites of transcription suggests that most splicing factors are indeed recruited from speckles (Fig. 4). It is possible that the origin of recruited splicing factors is dependent on the expression level of the transcribed gene. Although the nucleoplasmic pool of active splicing factors might be sufficient to splice transcripts of lesser abundance, additional factors might be required from nearby speckles to ensure efficient splicing of more highly expressed transcripts⁹. The peripheral movements and the (dis)association of particles seen in normally growing cells may represent the constant supply of splicing factors from speckles to sites of constitutively active genes. Regarding the mechanism of recruitment, we find that the dynamic events are sensitive to inhibitors of kinases and Ser/Thr phosphatases. Previously, two splicing-factor-specific kinases, SRPK-1 and CLK/STY, and a protein-phosphatase-1-like activity have been implicated in modulating the subnuclear distribution of several pre-mRNA splicing factors^{13,14,19}. Given that the two kinases belong to the LAMMER family of signal transduction kinases¹⁴, nuclear processes may be coordinated by signal transduction events.

Taken together, our results demonstrate the existence of dynamic events within the mammalian cell nucleus in living cells. Our observations show that pre-mRNA splicing factors respond specifically to gene activation by dynamic redistribution to the site of transcription. We suggest that these dynamic events reflect the spatial organization of transcription and pre-mRNA splicing, and that they are instrumental in the coordination of these two essential processes to ensure proper gene expression within the cell nucleus. □

Methods

Construction and characterization of fusion protein. SF2/ASF cDNA (provided by A. Krainer) was fused by PCR amplification in-frame upstream of GFP in pGFP-N1 (Clontech). BHK cells were transfected as described⁹. GFP-SF2/ASF was on average about 10-fold overexpressed. Indirect immunofluorescence was performed as described¹⁹. Mouse monoclonal antibody against U2-B'' (ref. 29) was used at a dilution of 1:4; auto-immune serum (provided by J. Craft) against the human Sm epitope was used at 1:2,500. For western blotting, cell lysates were prepared 14 h post-transfection and blotted as described¹⁹. Monoclonal mouse anti-SF2/ASF antibody recognizing RRM1 of SF2/ASF (A. Hanamura and A. Krainer, unpublished) was used for western blotting at 1:5. For analysis of the phosphorylation state of SF2/ASF and GFP-SF2/ASF, lysates were treated for 30 min at 37°C with 500 U ml⁻¹ alkaline phosphatase (New England Biolabs). The *in vivo* alternative splice-site selection assay was performed in HeLa cells 24 h after transfection⁷.

Time-lapse microscopy. Transfected cells were seeded onto glass coverslips and grown for 14 h. Coverslips were fitted into a FCS2 live-cell microscopy chamber (Bioptechs). The temperature of the cell chamber and medium was

kept constant at 37°C, and fresh DMEM supplemented with 10% fetal calf serum and 15 mM HEPES, pH 7.2, was perfused into the chamber every 30–60 min. The FCS2 chamber was mounted on a Zeiss Axiovert 405M inverted microscope equipped with a Photometrics Nu200 cooled CCD camera (1,320 × 1,035 array, 6.7 µm pixel size). For routine observation, a 100 × NA 1.3 oil-immersion lens was used. Images were acquired using Oncor Image 2.0.5 software. Exposure times were 0.2–0.8 s, and a GFP filter (Chroma Technology) was used. Images were pseudocoloured using the standard Oncor Image 2.0.5 pseudocolour look-up table. The spectrum of pseudocolours represents the intensities of the fluorescence signal measured for each pixel (grey level 0 in blue, grey level 255 in red). Time-lapse 'movies' were generated using Oncor Image 2.0.5 software. For quantification, a 'moving' speckle was defined as one that moved more than half its average diameter. A total of 16 cells from 4 experiments containing 368 speckles were analysed.

Drug treatments. We added 50 µg ml⁻¹ α-amanitin, 50 mM staurosporine or 1 µM okadaic acid to the medium. This concentration of staurosporine did not inhibit transcription, as demonstrated by normal incorporation of Br-UTP in cells grown for 1 h to 2 days in the presence of the drug.

Recruitment of GFP-SF2/ASF in living cells. BKT-1B cells (provided by T. Traavik) were grown in DMEM and stimulated by the addition of 100 µM dibutyryl cAMP (Sigma) as described²⁰. Rat R9 cells (provided by R. Dirks) were grown in DMEM and stimulated by the addition of 50 µg ml⁻¹ cycloheximide²². *In situ* hybridization using nick-translated biotinylated probes for the full-length BK virus DNA or the pSS plasmid was performed essentially as described²². Images of a particular series were aligned accurately using morphological markers such as shape of nuclei, position of nucleoli, and occasionally, cytoplasmic morphological features.

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Dimeric association and segmental variability in the structure of human CD4

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CD4 is a co-receptor in the cellular immune response. It increases the avidity of association between a T cell and an antigen-presenting cell by interacting with non-polymorphic portions of the complex between class II major histocompatibility complex (MHC) and T-cell receptor (TCR) molecules, and it contributes directly to signal transduction through its cytoplasmic association with the lymphocyte kinase Lck (ref. 1). CD4 also serves as the high-affinity receptor for cellular attachment and entry of the human immunodeficiency virus (HIV)². The extracellular portion of CD4 comprises four immunoglobulin-like domains (D1–D4). This part of human CD4 (residues 1–369) has been characterized as a recombinant soluble protein (sCD4)^{3,4}, and crystal structures have been described for the human D1D2 fragment^{5,6} and for the rat D3D4 fragment⁷. We have now determined the structures of intact sCD4 in three crystal lattices. These structures have a hinge-like variability at the D1D2 to D3D4 junction that might be important in immune recognition and HIV fusion, and a common dimeric association through D4 domains. Dynamic light scattering measurements and chemical crosslinking of sCD4 corroborate dimerization at high protein concentration. We suggest that such dimers may have relevance as mediators of signal transduction in T cells.

Crystals of sCD4 were grown as described previously⁴ except we started from the selenomethionyl protein. Moreover, the extent of measurable diffraction was increased through cryoprotection. The structure of a tetragonal type-C crystal was determined by molecular replacement⁸, confirmed by selenium Bijvoet-difference Fourier analyses, and refined with tight restraints at 3.9 Å resolution. This model was then used to solve the structures of selenomethionyl sCD4 in a new monoclinic type-F lattice and of native sCD4 in the trigonal type-A lattice. These structures were refined from rigid-body components only (Table 1). Although the results were obtained at modest resolution, they can be interpreted with

confidence in light of the detailed structures of the component domains^{7,9}. Density became resolved during refinement for the D1D2 to D3D4 connecting segment (residues 179–181) and for a carboxy-terminal extension (residues 362–363). These segments, as well as changes in sequence from rat to human D3D4 domain (58% identity), were modelled and refined. Residues 364–369 are not ordered in the crystals and may provide a flexible linkage to the transmembrane segment.

The six independent molecules in these crystals all have a similar extended structure (Fig. 1a), and in each crystal the two molecules per asymmetric unit are related by the same diad association of D4 domains (Fig. 1b). Lattice contacts elsewhere are distinctive (Fig. 1c), although one set of D1D2 contacts is similar in the tetragonal and monoclinic crystals. The D4–D4-associated dimers are butterfly-shaped in profile (Fig. 1c) with D1–D3 as 'wings', D4 pairs as the 'torso' and the C termini as 'legs', as if perching on a hypothetical membrane surface perpendicular to the diad axis. Each D1D2 fragment is a fixed entity at this resolution, as is each D3D4 dimer. There is, however, appreciable variability in the D1D2 to D3D4 junctions, which ranges up to 10.4° in orientational difference (Fig. 1d). The angle at the D1D2 to D3D4 junction is 140° (type-C molecule 1) as defined by the angle between the 'Cys-axes' (line through midpoints of canonical disulphide bonds or equivalents in neighbouring domains), and the D1D2 Cys-axis is 53° from the molecular diad axis and presumed membrane normal. The refined D3D4 component is very similar to the rat D3D4 model (1.07 and 0.96 Å r.m.s. deviations for Cα position in D3 and D4 with 5.4° relative difference in interdomain orientation).

The interface between D2 and D3 is formed almost exclusively by the AB loop of D2 and the FG loop of D3, both of which are more extended than their counterparts in D4 or D1, plus the connecting strand itself (Fig. 1a). There is 450 Å² of non-hydrated surface area buried from each domain into this interface, which is similar to the interfacial areas between D1 and D2 and between D3 and D4. Four hydrophobic residues (Leu 109, Leu 177, Leu 200 and Leu 283) dominate the interface. There is also an apparent hydrogen bond between Gln 112 and Gly 281. All of these are conserved residues. The last strand of D2 (G) continues directly into the first strand of D3 (A), but in a tortuous manner because of kinks in the A strand of D3 that switch the pairing from strand B initially to strand G later, as in most immunoglobulin-variable domains. The hinge flexibility at the junction is likely to be at residues Leu 177 and Ala 178 because Val 176 and Phe 179 are core elements of the D2 and D3 β-sandwiches, respectively. The junctional variability among the six sCD4 copies (Fig. 1d) is accommodated with little adjustment at the mostly hydrophobic interface; indeed, much larger movements toward more acute junction angles can be modelled without much steric hindrance. Such junctional flexibility is compatible with the results of the limited proteolysis experiments done on sCD4⁴.

The interface between protomers in the sCD4 dimer involves the D4 domains exclusively, and the buried surface (500 Å² from each non-hydrated protomer) is comparatively small, which suggests a relatively weak interaction. Although the operation that relates the protomers is non-crystallographic, it is within the bounds of error for a pure 2-fold axis ($\chi = 178.9^\circ$ and $t_x = 0.37$ Å after the type-C refinement). Both the CC' and FG loops of D4 protrude prominently from the C'CFG face of the β-sandwich⁷ and the diad interface involves these features in a manner reminiscent of clasped hands.

Table 1 Diffraction data and refinement

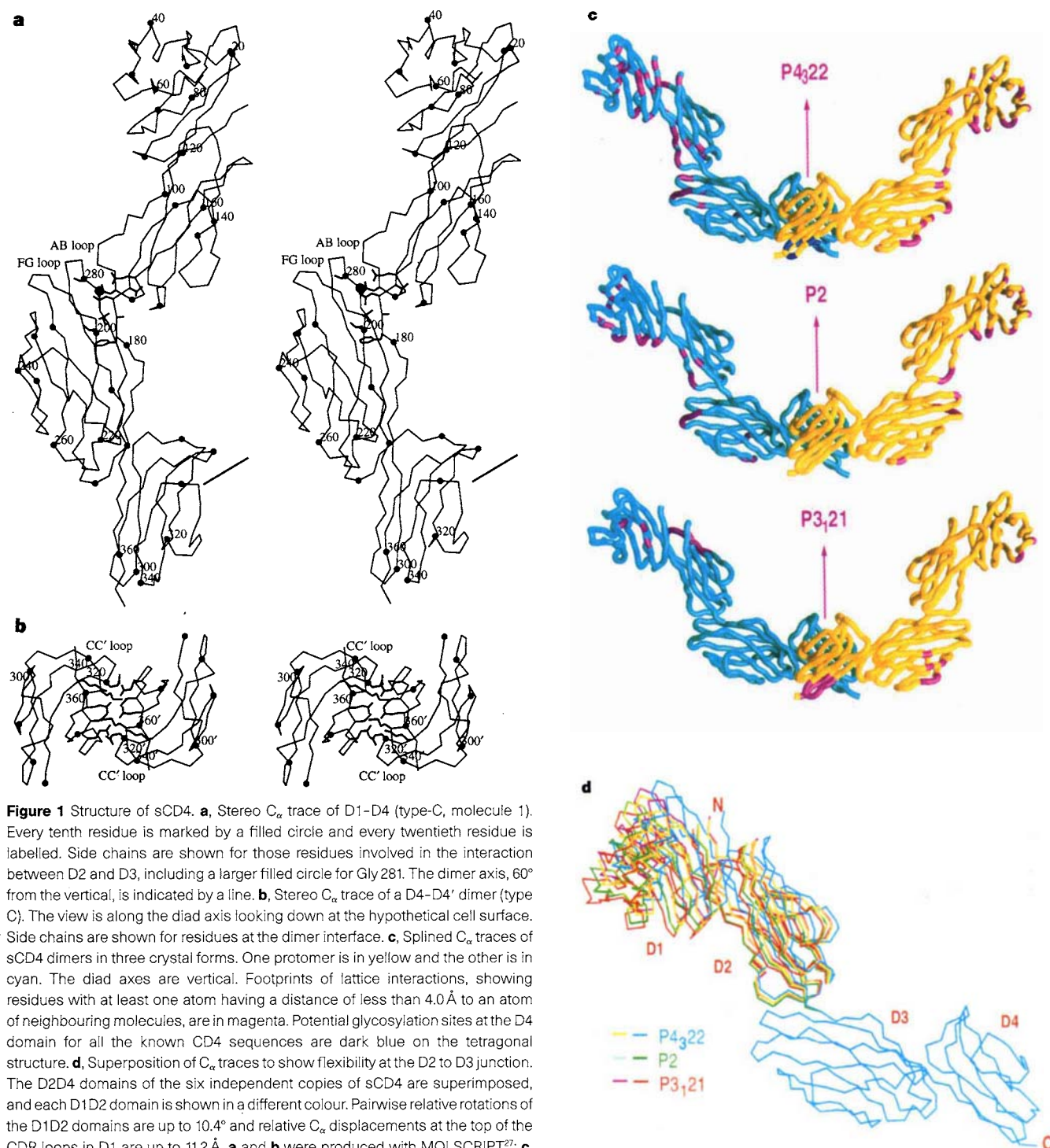
Crystal type	Space group	Copy/a.u.	Resolution (Å)	R_{merge}^* (%)	Completeness* (%)	Reflections	Rigid body R/R_{free} (%)	Refinement R/R_{free} (%)
C	P4 ₂ 2 ₂	2	8.0–3.9	11.2 (34.0)	80.0 (53.8)	11,993	41.2/42.5	24.0/35.1
F	P2 ₁	2	8.0–4.0	6.2 (25.4)	76.0 (45.8)	13,474	45.2/42.7	
A	P3 ₂ 2 ₁	2	8.0–5.0	8.6 (29.5)	67.3 (49.9)	4,289	42.2/44.2	

*Numbers in parentheses indicate statistics for highest resolution shells.

Here, residues from the F and G strands together with those at the CC' loop make direct contacts, and those at the FG loop may be close enough for water-mediated interactions (which cannot be modelled at this resolution). At the centre of the interface is a pair of absolutely conserved glutamine residues (Gln 344–Gln 344') separated at hydrogen-bonding distance. All possible glycosylation sites known from CD4 sequences map outside the D4–D4' interface (Fig. 1c) where they would neither interfere with nor participate in the dimer interaction. Although type-A crystals are in high salt and the C and F crystals are in low salt, all dimer interfaces are the same.

Given that dimers of sCD4 exist in these crystals, we have attempted to detect oligomerization of sCD4 in solution by way

of ultracentrifugation, light scattering and chemical crosslinking. Ultracentrifugation of sCD4 showed it to be predominantly monomeric at 1 mg ml^{-1} (ref. 4); at higher sCD4 concentration ($>5 \text{ mg ml}^{-1}$), however, non-ideal behaviour precluded satisfactory interpretation (P. Hensley, personal communication). Dynamic light-scattering measurements for D1D2 and sCD4 ($1.25\text{--}20 \text{ mg ml}^{-1}$) showed that the apparent hydrodynamic radius was nearly concentration independent for D1D2 but increased by 46% for sCD4, and is well beyond extremes of concentration dependence seen for non-associating species. Treatment of sCD4 with glutaraldehyde produced dimer bands in a concentration-dependent manner, whereas only monomers were



evident in similar experiments with D1D2, even at the molar concentration equivalent to the highest sCD4 concentration (Fig. 2). Quantification of dimer formation as a function of protein concentration could be fitted with a K_D of 44.3 mg ml^{-1} (1.08 mM) (Fig. 2). No analogous dimer was observed in the crystal of rat D3D4⁷, but the shorter construct of rat D3D4 (C terminus at residue 361, near the interface) may have interfered with dimerization. Moreover, with such low dimer affinity, dimers and monomers may coexist even under crystallization conditions so that favourable lattice contacts may compete successfully with the weak dimerization.

The crystalline sCD4 dimer is compatible with cellular assays of intact CD4 function for certain mutant variants. First, a mutational analysis of the interaction between CD4 and class II MHC found only one mutation outside domains D1 and D2 that had an effect¹⁰. This was located at residues 349–356 of human CD4, which overlap with the D4 dimer interface (Fig. 3). Second, an MHC-binding defective variant of CD4 (F43I) was shown to exert a dominant-negative effect on the ability of native CD4 to confer adherence to MHC-bearing cells and to boost T-cell activation, but only in the presence of its D3D4 domains, indicating oligomerization mediated by D3D4 (ref. 11). Moreover, the demonstration of two distinctive MHC sites for interaction with CD4 indicated the involvement of

oligomerization of CD4 and/or class II molecules into ordered arrays¹². Also, when the many point mutations in D1D2 that affect the MHC interaction^{10,13–15} are mapped onto the sCD4 surface (Fig. 3), it appears that each protomer must interact with two different MHC molecules thereby yielding network formation.

The observed junctional flexibility also seems to have functional consequences, both in immune function and in HIV infection. Mutations at the hinge abolish the normal CD4 enhancement of interleukin-2 production¹⁵. Deletion of the flexible linker between D4 and the membrane¹⁶ or binding of antibody Q425 near the D2 to D3 junction¹⁷ both block HIV-mediated membrane fusion without affecting gp120 binding. Presumably, the demonstrated association of the membrane-distal CD4-gp120 complex with the membrane-embedded chemokine co-receptor^{18,19} must require such flexibility.

CD4 is believed to increase the avidity of the TCR–MHC class II interaction which by itself would have typical lifetimes too short for effective signalling¹ and to contribute to signalling directly through its cytoplasmic association with Lck. The CD4–Lck association emulates a receptor tyrosine kinase, and we propose that ligand-induced dimerization²⁰ is the basis for signal transduction. In this case, the ligand is another cell. The C termini of the sCD4 dimer are $28 \text{ \AA}$ apart and project in parallel along the molecular diad. Kinase activation by *trans* autophosphorylation²¹ could ensue when CD4 dimers form. When evenly distributed on a T-cell surface the CD4 level (we estimate approximately 10^{-5} M) would favour monomers, whereas during antigen recognition CD4 will be recruited by cooperative interactions to the adhesion junction²² to a local concentration favouring dimers, especially when enhanced by the other interactions. In summary, the weak dimeric association of sCD4 seen first in the crystals and then confirmed in solution provides a basis for understanding signal transduction through CD4 in the cellular immune response. □

Methods

Protein preparation and crystallization. The expression, purification and crystallization of native sCD4 have been described earlier⁴. For selenomethionyl

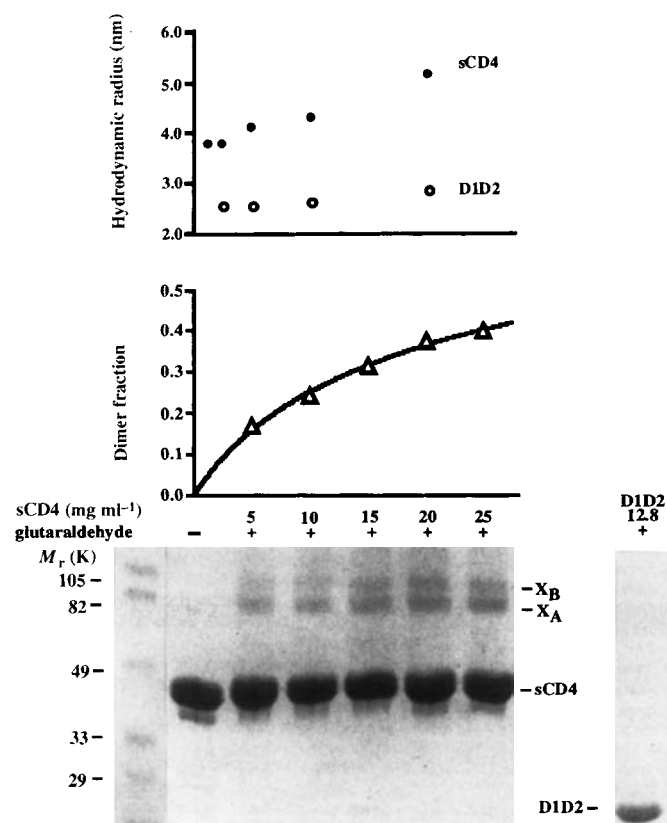


Figure 2 Solution experiments of sCD4. The top panel shows the apparent hydrodynamic radii as measured by dynamic light scattering of sCD4 (filled circles) at concentrations of 1.25, 2.5, 5.0, 10.0 and 20.0 mg ml^{-1} and of D1D2 (open circles) at concentrations of 2.5, 5.0, 10.0 and 20.0 mg ml^{-1} . The lower panels are results of crosslinking by glutaraldehyde. The SDS-PAGE gels contain molecular mass marker (lane 1), sCD4 (lane 2), sCD4 reacted with glutaraldehyde (lanes 3–7) with respective sCD4 concentrations of 5, 10, 15, 20 and 25 mg ml^{-1} and respective glutaraldehyde concentrations of 0.04, 0.08, 0.12, 0.16 and 0.20 mM, and D1D2 (lane 8) at 12.8 mg ml^{-1} reacted with 0.20 mM glutaraldehyde. Densities from the dimer bands as functions of protein concentrations were fitted to the dimerization equation with a single K_D (44.3 mg ml^{-1}) and a separate scale factor for each of the four repeated experiments. The average dimer fraction at each protein concentration is plotted together with the fitted curve. The r.m.s. residual in dimer fraction over the 20 independent observations was 0.02.

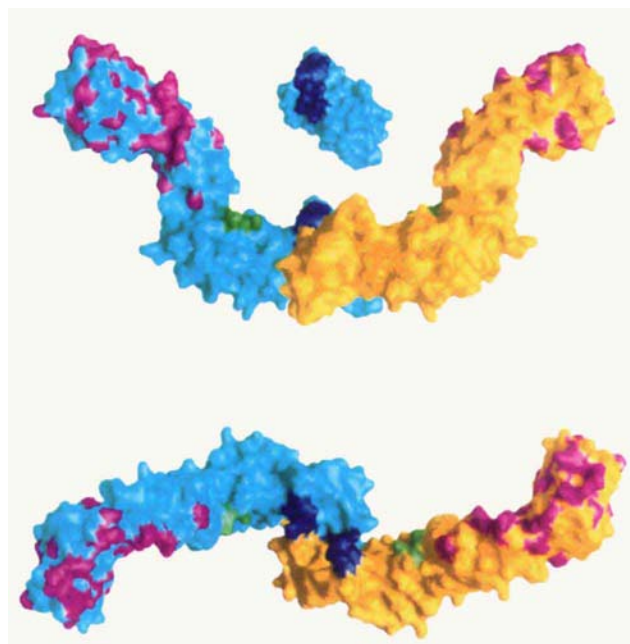


Figure 3 A summary of metagenesis data mapped onto the GRASP²⁸ surface of a sCD4 dimer. Solvent-exposed residues implicated in class II interaction by single-site mutations^{10,13–15} are shown in magenta. Residues on D3 that may interact with TCR components are in green; when mutated these had an effect only when assayed in association with TCR¹⁵. The stretch of residues in D4 that may affect dimerization of CD4 is in dark blue¹⁰. Two orthogonal views are shown, and one D4 domain is 'pulled' away to show the dimer interface.

sCD4 production, confluent Chinese hamster ovary (CHO) cells were adapted for 2 days in methionine-free media (25 mM selenomethionine) and then incubated for 3 days in fresh media containing 250 mM selenomethionine. The secreted selenomethionyl sCD4 (80% incorporation by amino-acid analysis) was captured on S-sepharose and immunoaffinity purified with the Ca^{2+} -dependent anti-CD4 antibody Q425 (ref. 17). Under crystallization conditions for native type-D crystals (PEG 400, pH 8.7, 4 °C)⁴, selenomethionyl crystals were obtained in the tetragonal type-C and the new monoclinic type-F lattices. To increase crystal size, sCD4 was pre-equilibrated for 4–5 days at 2/3 of the final PEG400 concentration (20%) in sitting-drop setups before dispensing into 4- μl hanging drops for final equilibrium. These crystals were directly cryoprotected in liquid propane. Type-A crystals were grown as before (ammonium phosphate, pH 8.7, 20 °C) from native sCD4 and used unfrozen. Cell dimensions for crystals used in the structure analyses are as follow: $a = b = 126.0$ Å and $c = 205.0$ Å for type A (trigonal); $a = b = 127.1$ Å and $c = 221.2$ Å for type C (tetragonal); and $a = 105$ Å, $b = 123.4$ Å, $c = 100.6$ Å, $\beta = 103.4^\circ$ for type F (monoclinic). Cell dimensions for the type-F lattice are similar to those of the type-D lattice⁴, but these crystals have a very different morphology and, using quantitative analysis²³ as well as structural verification, two rather than four monomers per asymmetric unit are found.

Structure determination. Diffraction data for selenomethionyl type-C and type-F crystals were collected at 110K at the peak of selenium K-shell absorption (0.9792 Å) on beamlines X25 and X4A, respectively, of the National Synchrotron Light Source. Data for native trigonal crystals in the type-A lattice were measured at room temperature at the F1 beam line of the Cornell High Energy Synchrotron Source. A combined molecular replacement procedure⁸ was used first to solve the type-C structure. Two human D1D2 fragments⁶ were located, then two D3 domains from the rat D3D4 model⁷ (non-identical residues reduced to alanine) were found, and finally the D4 domains were included and refined by rigid-body adjustments. Non-crystallographic symmetry averaging was performed at 3.9 Å resolution with the DM program from the CCP4 suite followed by model building with O²⁴. Residues 179–181 and 362–363 were added and the rat sequence of D3D4 was replaced by the correct human sequence to complete a model for residues 1–363. Refinement²⁵ proceeded with tight non-crystallographic restraints and R_{free} monitoring. The type-A and type-F crystals were solved by molecular replacement from the refined type-C model. Selenium Bijvoet-difference Fourier syntheses with model phases confirmed solutions for the C and F crystals; at least seven highest difference peaks corresponded to the expected eight selenium positions in each asymmetric unit.

Chemical crosslinking. Samples of sCD4 and D1D2 were reacted to completion (overnight) with glutaraldehyde at room temperature at constant molar ratios of protein to glutaraldehyde (3:1). The low glutaraldehyde concentration was chosen after a survey for conditions to prevent adventitious crosslinking at the higher concentrations of sCD4, D1D2 and bovine serum albumin (BSA). Each sample was denatured with 5% SDS and 2.5% β -mercaptoethanol, and a 3 μg aliquot was loaded and electrophoresed in 12.5% SDS polyacrylamide gel. Gels were stained by Coomassie blue and quantified with a densitometer. Staining is proportional to protein quantity within this range of densities. The crosslinking of sCD4 produced two higher molecular mass bands of approximately equal density that probably represent two possible linkage sites in an sCD4 dimer. The sCD4 model suggests two candidates for the observed species, one from Lys 360 to Lys 360' and another from Lys 322 to Lys 188', or vice versa; N ϵ atoms for these pairs are close (<8.0 Å) and accessible. Crosslinking was not observable for the D1D2 or BSA samples.

Each lysine residue should have its own characteristic probability of initial reaction with glutaraldehyde, independent of sCD4 concentration. This should be unaffected by neighbouring reactions here, because sCD4 hits will be rare with 39 lysine residues per sCD4 and a 3:1 protein to glutaraldehyde ratio. In the event of reaction at a crosslinking site in the dimer, we presume that the second step, which is intramolecular, will be committed. Therefore, the abundance of crosslinked species is proportional to the dimer fraction in the sample. Based on a dimerization model of $A + A = A_2$, the fraction of dimers in a given concentration of total protein (c_T) is given by

$$y = 1 - \frac{K_D}{4c_T} \left(\sqrt{1 + \frac{8c_T}{K_D}} - 1 \right)$$

where K_D is the dissociation constant. The dimer band density (D) is proportional to the dimer fraction, $D = S \times y$, where S is a scaling factor. A least-squares fitting of the $D(c_T)$ data give estimates of S and K_D .

Dynamic light scattering. Hydrodynamic radii of sCD4 and D1D2 at different concentrations were derived from dynamic light-scattering measurements²⁶ on a DynoPro Molecular Sizing Instrument (Protein Solutions, Inc.). Control experiments with BSA showed less than 6% variation over concentrations of 2.5–40 mg ml⁻¹.

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From Boehringer Mannheim Biochemicals
The company's first chemiluminescent imaging instrument has been produced in collaboration with Photometrics and Rodenstock

This user-friendly system should provide accurate quantification and digital analysis in a variety of non-radioactive, chemiluminescent techniques. Careful optimization of its high-performance CCD (charge-coupled device) camera, the optical system and its Windows LumiAnalyst software is said to result in simple robust performance. The technical improvement behind the Lumi-Imager is evident in the high-performance CCD chip sourced from Photometrics, according to the company. Its 1.3 megapixel resolution of $1,280 \times 1,024$ is stated to be over four times more powerful than previous cameras. Other features include a Rodenstock-supplied, focus-stabilized lens, which together with automatic software correction systems, provides both high sensitivity and accurate, reproducible data. The Peltier-cooled CCD camera and focus-stable lens, housed within a miniature darkroom enclosure, are the core of the Lumi-Imager. The system is said to eliminate tedious focusing or changing of lenses. Simple sample loading through a front-opening drawer is the only manipulation required. The complete workstation includes a Hewlett-Packard computer comprising a Pentium PC computer system, a monitor and a printer.

Reader Enquiry No. 100

ADC2250 analyser

From Dynamax

A soil respiration measurement system for ecology studies

The respiration rate in soil samples can now be analysed with the ADC2250 analyser. Using a flow meter board, the system has two integral input channels, allowing up to 24 soil samples to be monitored with multiplexing units. Measurements of carbon dioxide



ADC2250 analyses respiration of soil microflora.

(CO₂) evolution from soil microflora are needed for agrochemical and soil ecology experiments. Data can be collected for important toxicity tests, necessary for commercial agrochemicals to be registered with a carbon dioxide sensitivity of 0.1 p.p.m. Moreover, data from other sources, such as soil temperature and moisture, can be displayed and stored in the analyser.

Reader Enquiry No. 101

TSP UV6000LP detector

From Thermo Separation Products

A photodiode-array detector, incorporating the LightPipe flowcell design

According to the manufacturer, the sensitivity of this detector is five times that of previous photodiode array detectors. This technology is designed to solve sensitivity limitations. Using dual-light sources and a 20-bit analogue-to-digital converter, the detector is stated to deliver a 400 per cent increase in sensitivity. The optical pathlength has been extended with negligible light loss, enhancing the signal and reducing noise. At 50 mm, the optical path is five times that of a typical 10-mm pathlength. Transmitted light is channelled through the flowcell, the interior surface of which is lined with a chemically inert coating that has a refractive index lower than that for HPLC mobile phases. Due to total internal reflection, divergent light is redirected back into the flowcell's bore so that no light is absorbed by the flowcell's interior wall. The volume of the flowcell is only two picolitres per 10 mm of pathlength, reducing chromatographic band spreading for narrower, taller peaks. The instrument employs a fibre-optic beam to collect the circular cone of light exiting the flowcell and redistributes it into a thin slit, which accurately projects the emerging light onto the grating.

Reader Enquiry No. 102

Diamond ATR

From ASI

Designed to speed analysis of liquids, even corrosive and oxidizing materials, using FT-IR

SensIR technology provides a scratch-proof surface for sampling, such that the addition of a small amount of sample followed by wiping the sensor clean is all that is required for accurate and reproducible analysis. There are no cells to fill, no windows to polish, no ATR crystal to get scratched, and no need to mix or grind the sample with KBr. The ATR element in the DuraSamplIR is diamond, providing the most abrasion- and chemical-resistant



ASI's diamond-surfaced ATR resists chemicals and abrasion, and allows more efficient analysis.

sampling surface available. It is now possible to analyse liquids quickly and accurately without the normal concern of matching sample type to crystal material. Concerns over cleaning the ATR element are also said to be eliminated. The Diamond ATR eliminates the high cost of ATR crystal replacement and time consuming recalibration procedures.

Reader Enquiry No. 103

Phoenix microanalysis

From Edax

A Windows NT-based microanalysis system

The Phoenix microanalysis system offers multitasking and speed through the use of energy-dispersive microanalysis. The system includes digital-signal processing, higher speed X-ray mapping and imaging, higher resolution mapping and imaging (up to $4K \times 4K$), and is stated to provide reliable and accurate quantitative analysis without standards. The instrument uses EDAM III, the company's data acquisition module, and new PCI bus architecture to provide for the faster communication with the system's high-performance, dual 200-MHz PC workstation. It also uses a built-in digital signal processor for high count rate analysis and fast throughput, together with a newly designed scan generator for faster image acquisitions and higher quality X-ray maps. A built-in gated integrator also allows for improved images with lower



Edax's microanalysis optional SEM and TEM.

noise. To utilize the Windows NT environment fully, all software is written in native 32-bit code. Complete systems are available for both SEM and TEM applications, and utilize the company's standardless quantitative analysis routines.

Reader Enquiry No. 104

Spectramax Plus

From Molecular Devices

A spectrophotometer with the flexibility and high throughput of a microplate reader in a single system

The system provides ultraviolet/visible capability with an expanded wavelength range (190–1,000 nm); improved spectral resolution due to the 2-nm bandwidth; a wide dynamic range of zero to four absorbance units; the flexibility to use cuvettes, test tubes, or any combination of samples up to a full 96-well microplate; and the rapid reading 96 samples in as little as 10 seconds. Using a new technology called PathCheck, this instrument measures the actual pathlength of each sample in the microplate and normalizes the absorbance values to a 1-cm cuvette. Now, direct comparisons can be made between cuvettes and microplates, and assays can be easily converted to a high-throughput format while, at the same time, correcting for pipetting errors. PathCheck also makes it possible to determine the concentration of the sample directly, without a standard curve.

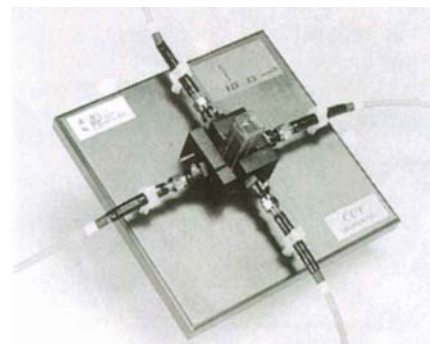
Reader Enquiry No. 105

Cuv-All

From Ocean Optics

A 1-cm cuvette holder that is equipped with fibre-optic couplings at four collimators

Optical fibres can be attached at these collimators to either read or illuminate the sample. Combined with Ocean Optics' modular spectrometers and light sources, the holder can measure absorbance, fluorescence, scattering or any combination of these optical phenomena. The four collimators include 5-mm diameter collimating lenses: two optimized for ultraviolet wavelengths and two for the visible. Each pair of lenses is posi-



Cuv-All cuvette holder with fibre-optic coupling.

tioned for 'straight-through' measurements, but is easily re-positioned to measure scattering or fluorescence. Adjustable barrels on each SMA-terminated collimator allow for precise focusing. The Cuv-All accepts 1-cm cuvettes and adjustable spring-loaded pistons hold the cuvette against two reference surfaces. The assembly also has a slot for holding filters of up to 6-mm thickness. In addition, the base plate has internal channels that can connect to a circulating, constant-temperature water source that provides temperature stabilization.

Reader Enquiry No. 106

Analysis and control

HP ChemAccess Client/Server

From Hewlett-Packard Europe

Designed to simplify the management of raw data, processed data and methods developed for laboratory instruments

Operating in a Windows 3.11, Windows95 or Windows NT environment, the client/server offers a single approach to instrument control, data evaluation, reporting and automation. It can be combined with the HP ChemStations for gas chromatography, liquid chromatography and capillary electrophoresis, offering centralized instrument control, which allows laboratory staff to control and monitor the status of instruments from their desktop PC. The software monitors the overall status of all laboratory instruments, which can be accessed directly from any remote PC linked to the server, allowing real-time plots to be displayed, for example. It also allows sequence and method control, remote macro execution and data organization tools to be used. Moreover, by allowing users to print data from other PCs linked to the server, ChemAccess also frees laboratory instruments from routine printing. This ensures that they can be dedicated to sample processing, increasing throughput. In addition, the system provides added security — log-on and passwords provide secure access to the network and server resources.

Reader Enquiry No. 107

BIAevaluation 3.0

From BIAcore

A new evaluation software for improved display and analysis of complex biomolecular interactions

According to the manufacturer, affinity and kinetics parameters are obtained in just minutes for a wide range of interaction mechanisms. The software includes advanced solutions for data evaluation, such as global fit, numerical integration and wizards. Users can select from a broad range of preprogrammed kinetic and affinity models or create their own. The system has a built-in simulator that generates theoretical interaction data for all models. Furthermore, it

fully supports concentration measurements, including the possibility to determine active concentration using the initial binding. BIAevaluation 3.0 is included with all new systems and is available for all current users of BIAcore biomolecular interaction analysis systems.

Reader Enquiry No. 108

Spectral Database on the Web

From Galactic

A free spectral archive database containing over 3,800 compounds in the 'Spectra Etc.' section of their Web site <www.galactic.com>.

Users can query the database by CAS number, compound name, molecular formula, or by FT-IR, ¹H NMR, ¹³C NMR and mass spectroscopy techniques. Database queries return the spectra, CAS number, molecular formula, compound name, original format, collection date, technique, source collection and resolution of the spectrum. Galactic's Web software renders the spectrum directly on the HTML page so that no helper application is needed to view spectra. The displayed spectrum can be 'zoomed' in on and scaled. Spectra can be downloaded in the Galactic SPC format for viewing and printing with the Galactic Data Viewer, which is also available for downloading off the Web or for data processing with GRAMS/32 or any other Galactic product.

Reader Enquiry No. 109

UV-Vis spectrometer temperature probe

From Unicam

A new in-cell temperature probe accessory works with the company's UV series and Helios spectrometers, as well as others

The probe has the advantage of measuring the temperature directly in the cell, and does not rely on the output from a temperature controller. Accurate temperature measurements are critical for many ultraviolet/visible applications in the biochemical and clinical markets, for example enzyme kinetics or DNA melting. The controller has three channels, enabling the measurement and logging of temperatures from three separate probes. The probe has a range of 0–100 °C, ±0.2 °C. The Windows-based software works with Windows95 only and allows the export of the measured temperature and the absolute time. These can be copied via the clipboard into any suitable reporting software, for example, Microsoft Excel. The temperature can then be graphically plotted against absorbance or time.

Reader Enquiry No. 110

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.